CEREAL CHEMISTRY

Published bi-monthly by

The American Association of Cereal Chemists

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Entered as second class matter at the post office at Minneapolis, Minn., under the Act of August 24, 1912. Acceptance for mailing at special rate of postage provided for in paragraph (d-2), Section 34.40, P. L. & R. of 1948, authorized February 16, 1934. Published at 500 So. 5th St., Minneapolis, Minn.

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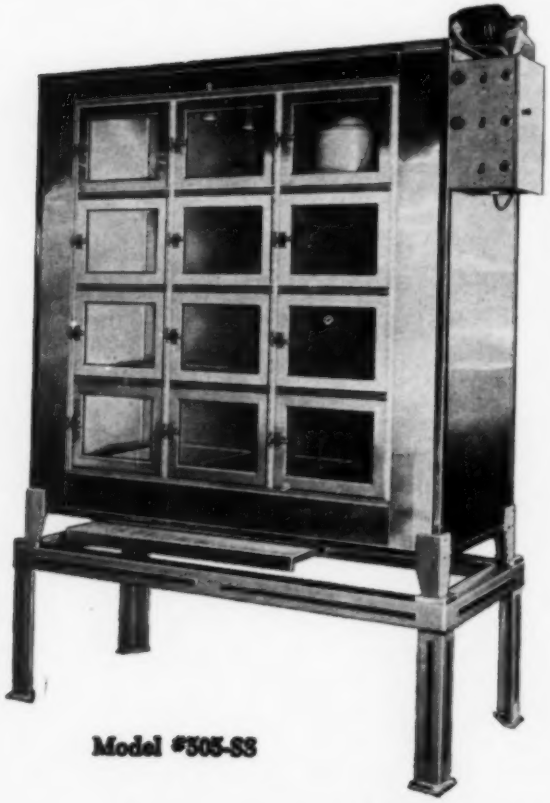

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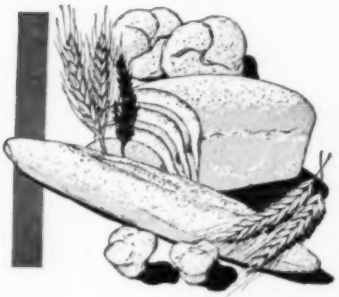

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CHAPTER VIII. *European Millwrights*

LEARNING BY DOING

The early European millwrights were great travelers. This was necessary because they were obliged to go where work offered. When they completed one job they moved on to the next. These men used their skills to design and then build the mills.

They learned by doing—and they taught as they traveled. Their knowledge was not recorded in books because they could neither read nor write. Still they taught. What they observed they passed on through maxims and by instructions to their apprentices.

Even with little knowledge of the forces involved they built mills that worked. They may not have known *why* they worked; they knew only that they *did* work. These untutored men served a valuable function by creating mills and improving the milling process. Man had long since learned that he could not eat wheatberries —that he *must* put grain through a mill to remove the rough, coarse bran particles and leave only the more desirable edible portion.

Sir Isaac Newton

SCIENCE ENTERS

As knowledge increased so did rational thinking. Traditional ways were examined and better ways evolved. Mathematics, long an abstract science, became a working tool. Measurements, proportions, calculations, ratios were developed into formulas which governed the millwright's work.

During the time that the mechanical skills were growing, other men of a different character were working to discover the scientific principles behind many mechanical devices. In the 16th century much work in practical mechanics was followed in the 17th by study in physical theory.

SCIENCE AND MECHANICS JOIN FORCES

Gradually science and mechanics grew closer together to their mutual benefit. The practical men, the millwrights, did not immediately accept the idea that scientific thought could be helpful. One, an Englishman, wrote in 1818 that advances were made only as a result of practical experience.

A different attitude was taken by another Englishman at about the same time who acknowledged that those skilled in science often lacked practical knowledge but said that the reverse was also true. Even so, the practical man — the millwright—and the theoretician did eventually form an alliance.

A NEW SOCIETY IS FORMED

Practical and theoretical knowledge increased. Mills grew in size. Production became greater. Wider markets were served. And a business society evolved. Along the way the artisan millwright disappeared for a good reason. As the mills became larger and more complex, investment became heavy. Too much was involved to be trusted to simple rule-of-thumb methods. More and more the practical man became guided by the findings of the scientist.

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The next chapter titled: "Milling in the Early American Colonies" will be published soon.

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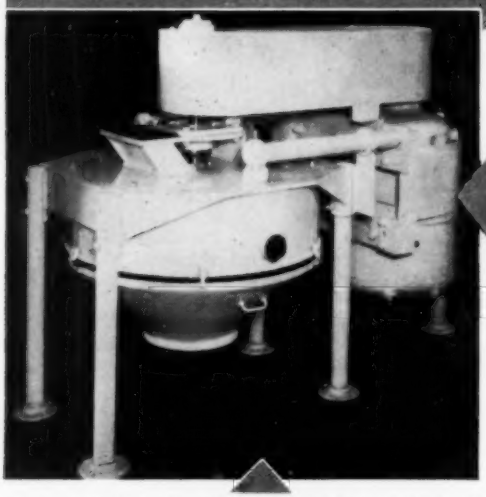

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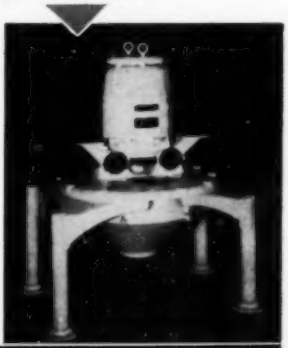

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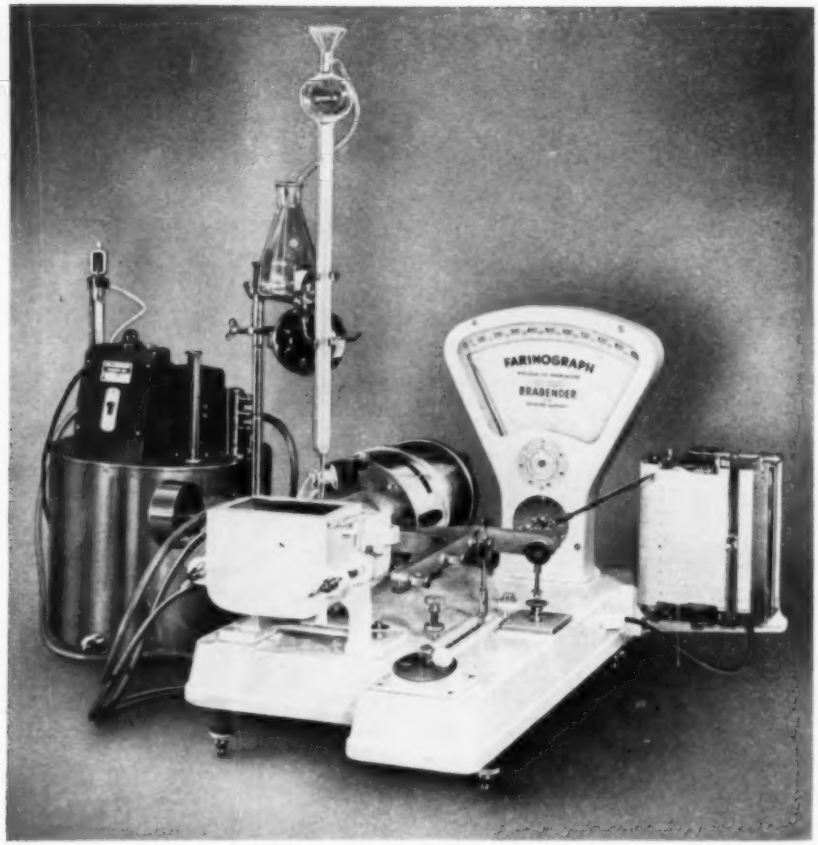

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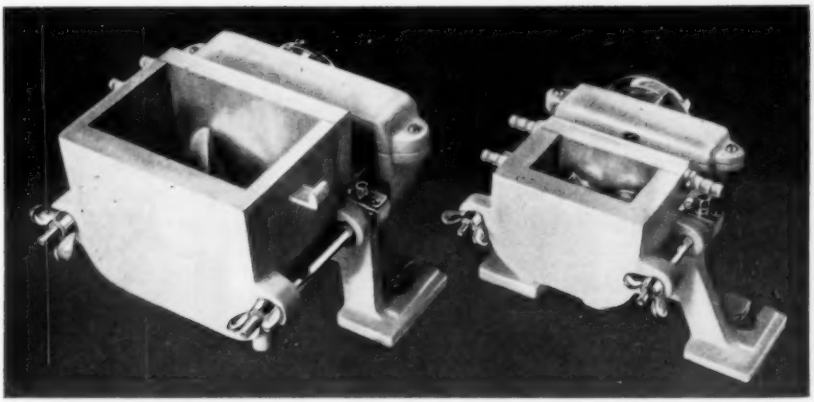

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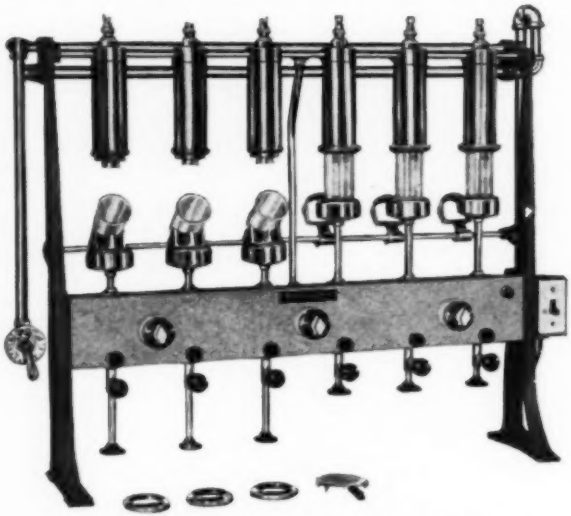

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CEREAL CHEMISTRY

VOL. 33 JANUARY, 1956 NO. 1

PROPERTIES OF CERTAIN PROTEASE SYSTEMS USED IN BREADMAKING[1]

JOHN A. JOHNSON,[2] BYRON S. MILLER,[3] P. D. BOYER,[4] and W. F. GEDDES[4]

ABSTRACT

Undenatured gluten was less readily hydrolyzed than hemoglobin by the proteases of malted wheat flour and of a fungus, *Aspergillus oryzae*. Denatured gluten was even less available to protease attack primarily because of its low solubility. The affinities of the proteases for gluten were greater than those for hemoglobin, although the rate of decomposition of the enzyme-substrate complex was greater for hemoglobin than for gluten. The *apparent* activation energies calculated for several enzyme systems were similar for hydrolysis of gluten, but variable for hydrolysis of hemoglobin. This indicates that similar peptide bonds were hydrolyzed by the different protease systems acting on gluten. The *apparent* activation energy value for hydrolysis of gluten in a sponge dough by fungal protease was 11,300 calories, which agreed well with that calculated for hydrolysis of undenatured dried gluten.

The pH optima were similar for different proteases acting on the same substrate, but the optima shifted for different substrates. The pH optima for malted wheat flour and fungal protease were 3.0–4.0 for gluten and hemoglobin, 5.5–6.0 for casein, and 7.0 for gelatin. Trypsin exhibited pH optima at 7.0–7.5 for hydrolysis of all substrates. Optimal pH values for hydrolysis of complex substrates appeared to be primarily a function of solubility and subsequent availability of the linkages to attack.

The malted wheat flour and fungal supplements contained enzymes that exhibited trypsin-like and dipeptidase-like activity but no pepsin-like or carboxypeptidase-like activity. The dipeptidase-like activity of the fungal protease system was destroyed by acid and heat treatment, while the trypsin-like activity was unaffected. Many specific peptide linkages not demonstrated by this investigation are very likely hydrolyzed by the protease systems of cereal and fungal supplements.

Interest in the use of proteases in breadmaking has been stimulated greatly by the introduction in 1950 (32) of fungal enzyme concentrates prepared from cultures of *Aspergillus oryzae*. They have high protease as well as alpha-amylase activity, and their use in sponge doughs reduces mixing requirements at the dough stage. Pro-

[1] Manuscript received August 18, 1955. Presented at the annual meeting, St. Louis, Missouri, May 1955. Condensed from the thesis submitted to the Graduate School of the University of Minnesota by John A. Johnson, in partial fulfillment of requirements for the Ph.D. degree, December, 1954. Contribution No. 259, Kansas Agricultural Experiment Station, Manhattan, Kansas. Scientific Journal Series No. 3460, Minnesota Agricultural Experiment Station, St. Paul, Minnesota.

[2] Professor, Kansas State College, Manhattan, Kansas.

[3] Chemist, Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

[4] Professor and Head, respectively, Department of Agricultural Biochemistry, University of Minnesota, St. Paul.

tease-treated doughs are more extensible and may be sheeted thinly and molded with greater ease. These effects are particularly beneficial in the case of "bucky" doughs.

There are two common sources of proteases used in baking: malted wheat and barley flour, and fungal preparations. The proteases of malted wheat flour were studied extensively by Mounfield (29, 30, 31). One was an endopeptidase as demonstrated by the cleavage of edestin (optimum pH, 4.1). Dipeptidase activity was evidenced by the hydrolysis of leucylglycine and glycylglycine (optimum pH, 7.3–7.9). Mounfield concluded that the degradation of edestin by malted wheat flour protease was accomplished by a series of consecutive reactions, each catalyzed by different proteases.

There is considerable variation in the pH optima found by different workers for the proteases of malted wheat flour. Mounfield (31) reported pH optima of 5.1 and 6.4 for the hydrolysis of gelatin and gluten, respectively. Koch and Ferrari (19) found the optimum pH range to be 6.8–7.0 when a viscometric gelatin assay procedure was used. On the other hand, Miller (25), employing Bacto hemoglobin as a substrate, reported an optimum pH of 3.5.

Little information exists on the properties of proteases from *Aspergillus oryzae* which are used in baking. Berger, Johnson, and Peterson (4) concluded that the extracellular proteolytic enzymes produced by this fungus consisted of at least one endopeptidase (optimum pH 7.0 with gelatin) and three exopeptidases (carboxypeptidase, amino polypeptidase, and dipeptidase). More recently Crewther and Lennox (8) also have demonstrated the heterogeneous nature of the protease enzymes elaborated by *A. oryzae*. They designated those components which acted primarily on proteins of high molecular weight as "viscometric" proteases, whereas those which degraded proteins of low molecular weight were called "gravimetric" proteases. The viscometric proteases, which exhibited optimum activities at pH 7.0, 7.5, and 10.0, were slightly inhibited by oxidizing agents such as iodine and hydrogen peroxide and were activated by cysteine.

Studies by several workers (9, 22, 23, 40) indicate that the amounts of the proteases vary in different species of fungi. McConnell *et al.* (23) found that fungi possess varying levels of amino peptidase, carboxypeptidase, and dipeptidase activity. The pH optima appeared to be more characteristic of the substrate than of the protease system.

The present study was undertaken to secure additional information on the factors influencing hydrolysis of gluten and hemoglobin by cereal and fungal proteases and to study the heterogeneity of these enzyme supplements.

Materials

Enzyme Preparations. The ten protease enzyme sources used included a patent flour, wheat germ, crude papain, malted wheat flour, malted barley, crude pepsin, trypsin, a purified barley concentrate (Colase), and the fungal enzymes Rhozyme-S and Rhozyme P-11.[5] All are of plant origin except trypsin and pepsin. Rhozyme-S was produced from *Aspergillus oryzae* and had a higher ratio of amylase to protease than did Rhozyme P-11. Protease (27) and alpha-amylase activities (36) of these enzyme sources, summarized in Table I, show wide variations. Since the pH optima for trypsin and pepsin are considerably different than pH 4.7 used for assay, their activities are not listed. The fungal preparations gave the highest values.

TABLE I

SUMMARY OF PROTEASE ACTIVITY OF VARIOUS ENZYME SOURCES

Enzyme Source	Protease[a, b]	Alpha-Amylase[c]
	Units/g	*Units/g*
Patent flour	1.04	.....
Wheat germ	23.2	.....
Malted wheat flour	49.7	72.0
Malted barley flour	65.4	185.0
Colase	555	3200.0
Papain	2820	.....
Rhozyme P-11	14060	119.0
Rhozyme-S	26440	4000.0

[a] Hemoglobin substrate, pH 4.7, and digestion time of 5 hours.
[b] See reference 27.
[c] See reference 36.

Buffer Systems. Different buffer systems were used, depending on the pH range employed. The concentrations of salts in the buffers were 0.5*M*, but in some instances they were increased to 1.0*M* to maintain a desired pH in the presence of the substrate. To maintain the pH within the ranges of 4.2–6.8 and 5.0–8.0, 0.5*M* sodium acetate-acetic acid and disodium hydrogen phosphate-monopotassium dihydrogen phosphate buffer solutions (13) were employed. Acetic acid was used to adjust the pH of the protein substrates within 3.0 to 4.0 and also to adjust the pH of dough in studies employing the farinograph. The pH values of the reaction mixtures were ascertained both at the beginning and end of the digestion period using a Beckman Model H-2

[5] Crude papain, crude pepsin, and trypsin from Merck & Co., Rahway, N. J.; malted wheat flour from Kansas Milling Co., Wichita, Kans.; malted barley from Kurth Malting Co., Milwaukee, Wis.; Colase from Columbia Malting Co., Chicago, Ill.; and Rhozyme-S and Rhozyme P-11 from Rohm and Haas Co., Philadelphia, Pa.

pH meter. In general, the buffer systems held the pH quite constant during a 5-hour digestion period. In the instance of the highest hydrogen-ion concentrations, there was some tendency for the pH to increase. The values accepted for comparison were those taken at the end of the digestion time.

Substrates. Bacto hemoglobin, undenatured wheat gluten, casein, and gelatin were used as substrates[6] where protease activity was assayed by determining the nitrogenous compounds not precipitated by trichloroacetic acid. The undenatured gluten was prepared from unbleached flour by vacuum drying at the Northern Utilization Research Branch Laboratory, Peoria, Illinois. Denatured gluten was prepared by drying wet crude gluten in an oven at 150° C. for 4 hours. The glutens were ground in a hammer mill to pass an 80-mesh silk sieve.

All synthetic peptides used to measure specificity, with the exception of carbobenzoxy-L-glutamyl-L-tyrosine, were purchased.[7] This substrate was prepared by reaction of carbobenzoxy-L-glutamic acid anhydride with the ethyl ester of L-tyrosine followed by de-esterification (6). The carbobenzoxy-L-glutamic acid anhydride was prepared from carbobenzoxy-L-glutamic acid, according to the method of Bergmann and Zervas (5).

Preparation of Enzyme Extracts. Enzyme solutions were prepared by stirring the dry preparations for 30 minutes at 30° C. with distilled water immediately prior to use. Malted wheat and barley flour extracts were centrifuged at 1800 r.p.m. for 5 minutes, and filtered through cotton following extraction. The enzyme concentrates, pepsin, trypsin, Colase, Rhozyme-S, and Rhozyme P-11, were filtered through Whatman No. 2 filter paper following extraction.

Procedures for Determining Protease Activity

Modified Ayre-Anderson Procedure. The modified Ayre-Anderson procedure used by Abbott, Miller, and Johnson (2) was employed as the basic procedure for the determination of proteolysis employing the various complex substrates. The quantities of hemoglobin, gluten, gelatin, and casein used per 50 ml. of reaction volume were 2.67, 2.0–3.0, 1.0, and 2.0 g., respectively. These substrate quantities provided an excess for all levels of enzymes used.

Unless the effect of temperature was being studied, 40°C. was the standard temperature for all assays. Likewise, unless the effect of hydro-

[6] Bacto hemoglobin from Difco Laboratories, Detroit, Mich.; casein and gelatin from Fisher Scientific Co., St. Louis, Mo.

[7] Glycylglycine, glycyl-DL-serine, glycyl-L-tryptophan, and carbobenzoxy-glycyl-L-phenylalanine from Mann Research Lab., Inc., New York 6, N.Y.; glycyl-L-tyrosine from General Biochemicals, Inc., Chagrin Falls, Ohio; leucyl-glycine from Nutritional Biochemicals Corp., Cleveland 28, Ohio; and benzoyl-L-arginine amide from H. M. Chemical Co., Ltd., Santa Monica, California.

gen-ion concentration was being studied, assays were performed at pH 4.7.

Alcoholic Titration Procedure. The alcoholic potassium hydroxide titration procedure of Grassman and Heyde (14) was employed for determining peptidase-like, pepsinase-like, and carboxypeptidase-like activity. The reaction mixture digested at 40° C. consisted of 1 ml. each of 0.05*M* substrate, enzyme, and buffer, and 0.001*M* potassium cyanide to inhibit the growth of microorganisms. Sixty-microliter aliquots were titrated after 8 hours, using a Rehberg buret (7) and a stream of nitrogen directed over the solution during titration to prevent absorption of carbon dioxide.

Trypsin-like activity was measured using a micro-diffusion technic (7, 39). Trypsin-like activity was measured by digesting 0.5 ml. of 0.05*M* benzoyl-L-arginine amide with the enzyme extract (0.5 ml. containing 0.003*M* potassium cyanide) in the presence of 0.5 ml. of buffer. After 8 hours at 37.8° C., 60μl. of 0.1*N* hydrochloric acid were added to the center well and 0.5 ml. of saturated sodium carbonate added to the outer ring. The glass cover was replaced and the cell returned to the incubator for 3 hours to allow diffusion of the libterated ammonia. Appropriate "blank" determinations were made simultaneously. Activity was expressed in μl. of 0.1*N* sodium hydroxide solution per mg. of enzyme nitrogen.

Results and Discussion

Traditional substrates for study of protease action are of animal origin and include casein, gelatin, and hemoglobin. Proteolysis in bread doughs involves splitting of a plant protein, gluten, which has been used by few investigators (2, 18, 31). One study that has included gluten as a substrate is that of Johnson and Miller (18), who reported on changes in dough consistency as a measure of proteolysis. It was deemed necessary, for this study, to obtain data comparing the action of proteases on gluten as well as a common substrate such as hemoglobin. Furthermore, it was desirable to study dried gluten prepared under specified conditions. Such a substrate may be used in the methods which normally employ animal proteins.

Comparison of the Proteolysis of Gluten and Hemoglobin by Fungal and Malted Wheat Flour Protease

Dried undenatured gluten was found to be less available to fungal and malted wheat flour protease action than hemoglobin. Typical rate constants calculated on the basis of a zero-order reaction for the hydrolysis of gluten and hemoglobin by Rhozyme-S and malted wheat

TABLE II

REACTION RATE CONSTANTS FOR HYDROLYSIS OF GLUTEN AND HEMOGLOBIN[a]

ENZYME	GLUTEN[b]	HEMOGLOBIN[b]
	mg.	*mg.*
Rhozyme-S	0.6×10^{-1}	1.4×10^{-1}
Malted wheat flour	1.0×10^{-1}	1.6×10^{-1}

[a] Conditions: pH 4.7; temperature 40°C.; initial substrate concentrations: gluten, 112.5 mg. nitrogen; hemoglobin, 413 mg. nitrogen.
[b] Mg. nitrogen released per minute.

flour are shown in Table II. The low activity on gluten was believed to be due partly to the insolubility of the substrate and the inability of the enzyme to find suitable sites for formation of the enzyme-substrate complex. Several attempts to modify gluten or increase its solubility did not change the rate of hydrolysis by malted wheat flour protease. These experiments included grinding the gluten in a ball mill for 48 hours and extracting the lipids with Skellysolve B followed by ethanol.

Denaturation of proteins generally decreases their solubility but may increase their availability to enzyme attack (33). Experiments were performed by subjecting denatured and undenatured gluten to hydrolysis by malted wheat flour protease (Fig. 1). The reduction in the extent of hydrolysis of denatured gluten can be attributed to insolubility, since the solubility of the protein in the acetate buffer (pH 4.7) was reduced 60% by denaturation.

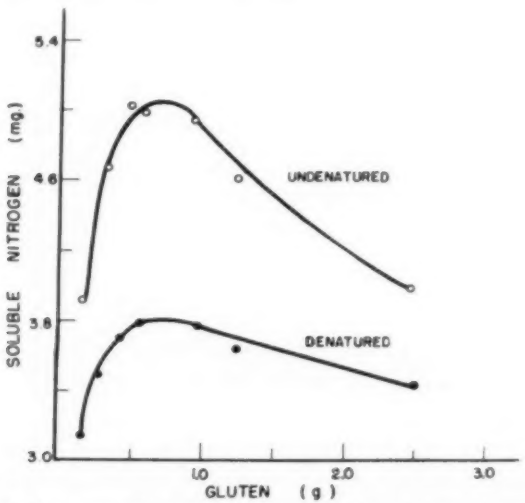

Fig. 1. The relationship between malted wheat flour protease activity and gluten substrate concentration.

Usually excess substrate is necessary for the accurate assay of enzyme potency. Under some conditions, however, high substrate concentrations may retard proteolysis (38). Experience with hemoglobin has shown that an excess of this substrate does not inhibit proteolysis. Excess gluten, however, when used as a substrate for malted wheat or barley flour protease, inhibited proteolysis (Fig. 1). Although the cause of this reduction is not clear, it is assumed to be due to the partial inhibition of the proteases by a component of the gluten. Such inhibition did not occur with Rhozyme-S, pepsin, unactivated papain, Colase, or malted wheat or barley flour extracts which were treated to remove the alpha-amylase (26). It appears that a specific protease of these malted flours might be inhibited. This may be the fraction which is inhibited by oxidants (18) and may or may not be inhibited by the papain inhibitor in flour (15).

These experiments demonstrated the necessity of using an optimum amount of substrate when gluten was hydrolyzed by malted wheat or barley proteases. They also demonstrated the desirability of increasing the amount of protease when using gluten over that required when hemoglobin was used as a substrate.

Although there was evidence to indicate that gluten is less available to protease action than hemoglobin, owing partially to differences in substrate solubility, it appeared of value to study the relative affinity of the enzymes for the substrates and the dissociation of this complex. The Michaelis-Menten constants (24) were obtained by use of the following equation derived from the Michaelis-Menten theory:

$$\frac{S}{v} = (1/Vm)\ S + \frac{Km}{Vm},$$

where S is the substrate concentration expressed in mg. of substrate nitrogen; v is the velocity expressed as mg. of soluble nitrogen released per minute; Km is the Michaelis-Menten constant; and Vm is the maximum velocity of reaction. A plot of S/v against S gave a straight line intercepting the ordinate at a point equal to Km/Vm. The slope of the line (1/Vm) was used to calculate Vm which was employed to evaluate Km, the unknown quantity in Km/Vm (20).

The dissociation of the enzyme-substrate complex into the products of digestion is proportional to the maximum velocity (Vm). The reversible dissociation of the complex also may be considered insignificant in cases where the individual constants have been measured directly. It may be hypothesized that the rate of formation of the enzyme-substrate complex is the same for both substrates, since the amount of enzyme is small compared to the large excess of substrate. For these

reasons some measure of the enzyme-substrate affinity and rate of dissociation of the complex can be obtained from consideration of the Michaelis-Menten constant (Km) and the maximum reaction velocity (Vm). Michaelis-Menten constants and maximum velocity values are presented in Table III for the hydrolysis of gluten and hemoglobin by Rhozyme-S and malted wheat flour protease. It is evident from

TABLE III

COMPARISON OF MICHAELIS-MENTEN CONSTANTS (KM) FOR RHOZYME-S AND MALTED WHEAT FLOUR ACTING ON GLUTEN AND BACTO HEMOGLOBIN [a]

ENZYME	ENZYME CONCENTRATION	SUBSTRATE	KM	VM
Rhozyme-S	10 mg.	Hemoglobin	99.2	32×10^{-2}
Rhozyme-S	10 mg.	Gluten	40.5	11.6×10^{-2}
Malted wheat flour	4 g	Hemoglobin	27.4	9.1×10^{-2}
Malted wheat flour	4 g	Gluten	18.3	4.6×10^{-2}

[a] Conditions: t = 300 minutes; pH 4.7; temperature 40°C. Substrate concentration ranged from 10.7 to 215 mg. for hemoglobin and 20.3 to 400 mg. protein nitrogen for gluten.

these data that the rate of decomposition of the enzyme-substrate complex, as shown by the Vm values, is greater for hemoglobin than for gluten. The affinity of the enzymes, as shown by the Km values, is greater for gluten than for hemoglobin.

The data in Tables II and III for the hydrolysis of gluten and hemoglobin indicate that gluten is hydrolyzed less rapidly than hemoglobin by fungal and malted wheat flour protease. It was therefore of interest to measure the effect of temperature on the rate of reaction. An excess (or optimum amount of gluten in the case of malted cereals) of gluten or hemoglobin was hydrolyzed at 25°, 30°, 35°, and 40°C. The activation energy values (E) were calculated according to the Arrhenius equation:

$$\log K = \frac{-E}{2.303\,R} \cdot \frac{1}{T} + C,$$

where K is the reaction rate constant; E is "apparent" activation energy in calories per mole; R is the gas constant, 1.98 calories; T is degrees absolute temperature; and C is a constant. The logarithms of the reaction rate constants were plotted against the reciprocal of the absolute temperatures, 1/T (37).

The activation energy values calculated for the hydrolysis of gluten and hemoglobin by impure enzyme systems do not represent the energy of activation for a single reaction. Nevertheless, they should serve to demonstrate differences in energy required to hydrolyze the peptide bonds of different substrates. The effect of temperature on

hydrolysis of gluten and hemoglobin by several enzyme systems is presented in Fig. 2. The activation energies calculated from the data in Fig. 2 are presented in Table IV. It is evident from these data that the effect of increasing the temperature on hydrolysis is similar for the two substrates.

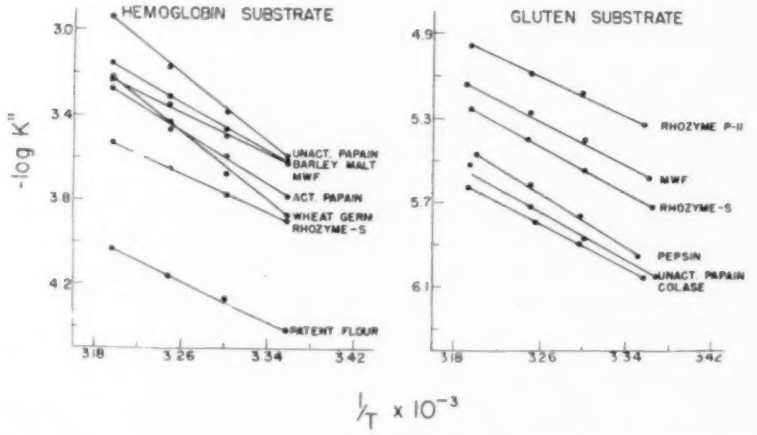

Fig. 2. Effect of temperature on the rate of hydrolysis of gluten and hemoglobin substrates by various protease systems.

TABLE IV

APPARENT ACTIVATION ENERGY VALUES FOR SEVERAL PROTEOLYTIC ENZYMES USING THE AYRE-ANDERSON METHOD OF ANALYSIS[a]
(Temperatures ranged from 25° to 40°C.)

ENZYME	SUBSTRATE	APPARENT ACTIVATION ENERGY VALUES
		calories/mole
Malted wheat flour	Gluten	12,400
Rhozyme-S	Gluten	12,500
Rhozyme P-11 (purified)	Gluten	10,700
Papain (unactivated)	Gluten	12,700
Pepsin	Gluten	13,900
Colase (barley malt, purified)	Gluten	12,700
Malted wheat flour	Hemoglobin	11,100
Rhozyme-S	Hemoglobin	10,500
Papain (unactivated)	Hemoglobin	18,500
Papain (activated)	Hemoglobin	14,200
Barley malt (crude)	Hemoglobin	12,600
Patent flour	Hemoglobin	10,700
Wheat germ	Hemoglobin	18,600

[a] See reference 2.

The close agreement of the activation energies for the different enzymes acting on gluten indicates that they have similar capacities for decreasing the energy required to hydrolyze the peptide bonds of gluten. The variation among the values encountered for different enzymes acting on hemoglobin contrasts with the similarity in values for gluten. This variation suggests that the different enzyme systems are hydrolyzing different peptide bonds of the hemoglobin molecule. The similarity of the values for hydrolysis of gluten suggest that similar bonds are split by the several enzyme systems. The values agree well with those reported for other proteases (37, 38).

The effect of temperature on protease activity in sponge doughs was investigated by allowing the protease to react with gluten in dough at temperatures ranging from 25° to 40°C. at pH 4.7 using an excess of alpha-amylase (18). When log K was plotted against 1/T, a straight-line function was obtained (Fig. 3) from which an *apparent* activation energy value of 11,300 calories per mole was calculated. This value compares favorably with those obtained by the Ayre-Anderson technic (Table III).

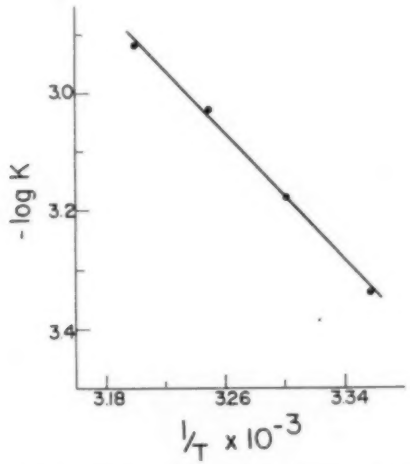

Fig. 3. Effect of temperature on changes in consistency of dough expressed as first-order reaction kinetics.

Effect of Hydrogen-Ion Concentration on Protease Activity

The hydrogen-ion concentration is known to affect both the protein substrate and the enzyme itself which also is a protein. The relative importance of these two effects is not generally known or appreciated.

This phase of the work was undertaken to obtain comparable data on the effect of pH on the proteolysis of several substrates by different proteases.

The results of pH-activity studies employing complex natural substrates are presented in Fig. 4. With the exception of trypsin, all

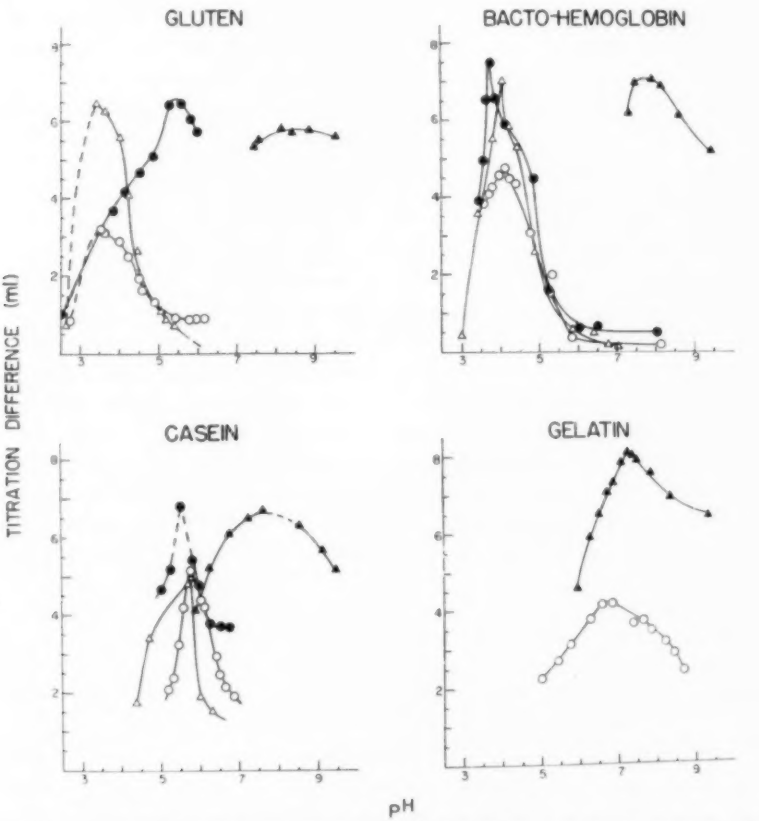

Fig. 4. Effect of hydrogen-ion concentration on hydrolysis of gluten, Bacto hemoglobin, casein, and gelatin by papain (●), malted wheat flour (△), Rhozyme-S (○), and trypsin (▲).

enzymes had pH optima that shifted, depending on the substrate. Thus, for malted wheat flour and fungal protease, the pH optima were at 3.0–4.0 for hydrolysis of gluten and Bacto hemoglobin and 5.5–6.0 for casein. Using gelatin as a substrate, the pH optima for fungal protease shifted to 7.0 compared with 3.5, 4.0, and 6.0 when gluten, Bacto hemoglobin, and casein were used, respectively.

The data for trypsin are of interest because this enzyme appeared to maintain a pH optimum near 7.0–8.0 irrespective of the substrate. In most instances, the optimum is represented by a broad plateau.

The pH optimum for papain was near 5.5 for gluten and casein substrates but it shifted to 3.7 when Bacto hemoglobin was used. These data do not agree with those of Lineweaver and Schwimmer (24), who found a pH optimum near 7.0 for crystalline papain. Since comparative experiments with and without added cysteine (10 mg. cysteine per mg. of papain) caused a decrease in the pH optimum of only 0.4, it must be presumed that differences in pH optima are not due to activation but rather that Lineweaver and Schwimmer were working with a certain crystalline fraction of the original crude papain. The pH values for trypsin are in general agreement with the accepted pH optima. The optimum pH values for wheat protease acting on casein are in good agreement with those of Howe and Glick (17) and those of Miller (25) for hydrolysis of Bacto hemoglobin.

The pH optimum for fungal protease acting on gelatin agrees with that found by Berger, Johnson, and Peterson (4). The pH optimum for papain acting on casein was slightly lower than those found by several investigators (3, 16, 21). The pH optimum for fungal protease does not agree with those reported by Wetter (40) and McConnell (22), but this may be expected, since these measurements were with different sources of fungal protease. The pH optimum found for malted wheat protease using gluten substrate is different from those reported by Mounfield (29, 31). This may be due to the fact that Mounfield employed the formol titration method to measure proteolysis. Mounfield showed that malted wheat flour contains a peptidase having a pH optimum between 7.3 and 7.9. In the measurement of proteolysis by the formol titration, the effect of peptidase activity may have contributed to the increase in carboxyl groups which was taken as a measure of proteolysis.

The reasons for variations in the effect of pH upon the activity of enzymes acting on various substrates are obscure. Part of the variation may be attributable to the various methods used to measure proteolysis. Assuming that the enzyme preparations contain different protease enzymes, a method designed primarily to measure endopeptidase would exhibit a different pH optimum from one designed to measure exopeptidase activity. The effect of the hydrogen-ion concentration on substrate solubility and its availability to enzyme attack may also be a factor, particularly with substrates such as gluten and hemoglobin, which become increasingly soluble or dispersed as the hydrogen-ion concentration is increased to pH 1.0–2.0. Since there may be concomi-

tant inactivation of the enzyme as the pH approaches 2.0, the apparent optimum activity of an enzyme may be somewhere between the pH of adequate dispersement and loss of activity of the enzyme due to inactivation.

Heterogeneity of Malted Wheat Flour and Fungal Proteases

The most direct method of measuring heterogeneity of a protease system is to employ synthetic substrates of known structure. The substrates studied were such as to show the presence or absence of specificity resembling that of pepsin, trypsin, carboxypeptidase, and certain dipeptidases.

Pepsin-like and Carboxypeptidase-like Activity. No pepsin-like or carboxypeptidase-like activity was found for either malted wheat flour or the fungal preparation. This was established by use of carbobenzoxy-glutamyl-L-tyrosine and carbobenzoxy-glycyl-L-phenylalanine, respectively. In these experiments several concentrations of enzymes were employed. In the instance of malted wheat flour, without recourse to purification procedures and possible loss of the particular enzyme being studied, the highest concentration that could be used conveniently was 1.0 ml. of a 1:2 extract per 1.0 ml. substrate (0.05*M*). A pH of 4.0 (11, 12) and digestion time of 24 hours were used in the presence of 0.001*M* potassium cyanide to inhibit microbiological growth. Similarly, for Rhozyme-S, the concentration was 20 mg. per test. On the basis of reaction with Bacto hemoglobin, 1 mg. of Rhozyme-S is equivalent to 500 mg. of malted wheat flour. Attempts also were made to utilize a partially purified malted wheat flour protease that was 120 times as active as normal malted wheat flour protease (50 units per g.) (1). This preparation also showed no pepsin-like or carboxypeptidase-like activity. However, it was not certain whether the carboxypeptidase was destroyed during purification. It was concluded that pepsin-like or carboxypeptidase-like activity was not present in the malted wheat flour or fungal supplements.

Trypsin-like and Dipeptidase-like Activity. Both enzyme preparations showed trypsin-like and dipeptidase-like activity by their reaction with benzoyl-L-arginine amide and several different dipeptide substrates (Fig. 5). All curves possess a rather broad peak. The level of activity in the fungal preparations is less than that for an equivalent amount of malted wheat flour, on the basis of its action on hemoglobin. Indeed, no activity of any kind could be established for the fungal preparation until the concentration was increased several-fold above that equivalent to malted wheat flour. No activity could be demonstrated for the fungal preparation acting on glycyl-L-tyrosine, but

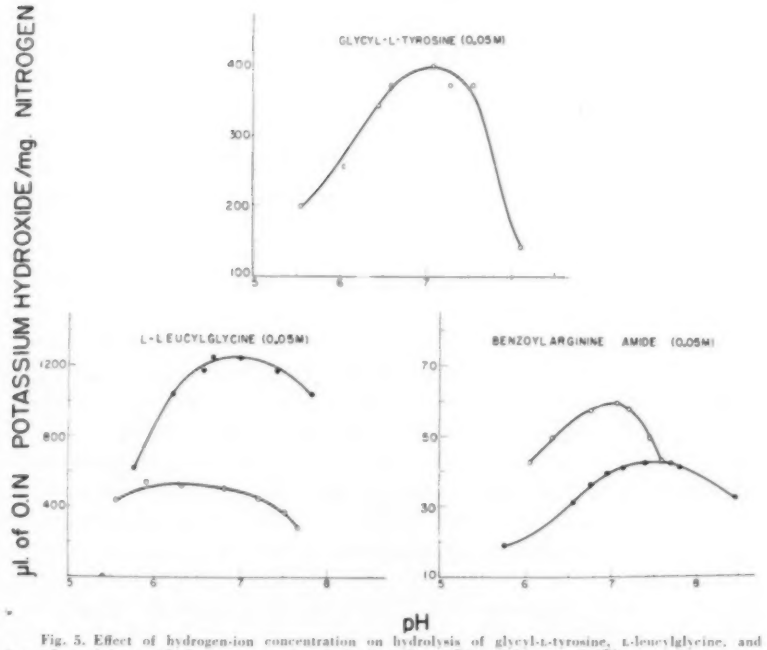

Fig. 5. Effect of hydrogen-ion concentration on hydrolysis of glycyl-L-tyrosine, L-leucylglycine, and benzoyl-L-arginine amide by proteases of malted wheat flour (○) and Rhozyme-S (●).

both the fungal and malted wheat flour protease systems split glycyl-DL-serine, glycyl-L-tryptophan, and glycylglycine substrates.

Effect of Heat Treatment on Trypsin-like and Dipeptidase-like Activity. The activity of the preparations treated by the technic of Miller and Johnson (26) to remove the alpha-amylase indicated that several of the specific peptide bond-splitting enzymes are inactivated by this procedure. The treatment to destroy alpha-amylase in the fungal preparation removed all specific types of proteolytic activity which were measured, except the trypsin-like activity which was maintained at full strength. The treatment to inactivate the alpha-amylase in the malted wheat flour caused no loss in the capacity of the malted wheat flour to split glycylglycine or glycyl-L-tryptophan, but a 60% decrease in trypsin-like activity was observed. All other types of activity demonstrated for the original malted wheat flour were completely eliminated by the acid and heat treatment.

Conclusions

Neither denatured nor undenatured gluten is as available to enzyme hydrolysis as hemoglobin. This may be due mainly to its insolubility.

The fungal and malted wheat flour proteases were shown to have a greater affinity for gluten than for hemoglobin, although the rate of decomposition of the enzyme substrate complex was greater for hemoglobin. These facts serve to illustrate some of the problems associated with the use of a dried gluten substrate to determine protease activity.

The similarity of the *apparent* activation energy values for hydrolysis of gluten by various enzyme systems suggests that the different enzyme systems hydrolyzed similar bonds. The variation encountered for hydrolysis of hemoglobin suggests that different bonds were split by the various systems. The activation energy for the hydrolysis of gluten in dough was similar to that determined for the same enzyme system hydrolyzing dried gluten. This indicates that similar peptide bonds were split in gluten of dough and undenatured dried gluten substrates.

Some material associated with, or a component part of, the gluten inhibited a fraction of the protease present in malted wheat or barley flour. Fungal enzymes were not affected similarly. These data suggest a fundamental difference between the proteases of fungal preparations and malted wheat flour.

Available data do not permit an evaluation of the relative importance of the effect of pH on substrate availability and its effect on the enzyme. Data obtained in this study indicate that pH optima, with the exception of that of trypsin, shift depending on the substrate employed. The pH optima for the various enzyme systems were similar when the same substrate was hydrolyzed. The pH optimum for trypsin was found to be 7.0–8.0, independent of the substrate. The present studies appear to support the contention of Northrop (34), Moelwyn-Hughes (28), and Fruton (10) that the pH optimum is influenced by the nature and concentration of the substrate.

Malted wheat flour and fungal preparations contained several proteases. Both sources exhibited trypsin-like activity, but malted wheat flour was more active with respect to this component. Several types of dipeptidase-like activity were demonstrated. No pepsin or carboxypeptidase-like activity could be demonstrated. These experiments do not demonstrate all of the types of protease that may be present in fungal and malted wheat flour preparations. In order to follow this problem to its logical conclusion, it would be necessary to perform end-group analysis on the products resulting from enzyme digestion. Such data could provide a logical basis for selecting synthetic substrates which would reveal the type of linkage split in hemoglobin, gluten, or other substrates. Such information might suggest the use of certain synthetic substrates for an accurate analysis of proteolytic enzyme systems used for a specific purpose.

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A MICROBIOLOGICAL METHOD FOR DETERMINATION OF CYSTINE IN FOODS[1]

MILLARD J. HORN AND AMOS E. BLUM

ABSTRACT

A new basal medium for the microbiological determination of cystine has been developed using an acid hydrolysate of a "lanthionized" casein plus tryptophan and arginine as a source of amino acids. This medium gives reproducible standard curves. A study of the rates of liberation of eleven amino acids from a protein and a cereal indicates no loss of amino acids on long hydrolysis except cystine. As a result of these experiments, 2-hour hydrolysis was used for all cystine assays. Data are presented on the cystine content of 60 proteins and foods.

Attempts have been made to obtain reliable standard curves for cystine, using a synthetic basal medium and hydrolysates of peroxide-treated casein (30) with *Leuconostoc mesenteroides* P-60 as the test organism. These attempts were unsuccessful. Peroxide-treated casein gave better results than the synthetic medium, but it was found difficult to duplicate the standard curve owing to inability to standardize the destruction of amino acids by the peroxide treatment.

A sample of "decystinized" casein prepared by Jones and Gersdorff (20), which was available in the laboratory, was hydrolyzed and used as a test basal medium. This hydrolysate gave excellent results but had the disadvantage that the preparation of the protein was long and tedious.

When lanthionine was discovered (17, 18, 19), it was found that proteins boiled with 2.00% sodium carbonate changed the cystine to lanthionine. This led to a study of the use of a "lanthionized" casein as a source of amino acids in a basal medium.

This paper describes the preparation of "lanthionized" casein hydrolysate and its use in the basal medium for the microbiological assay of cystine in 60 proteins and foods.

Materials and Methods

This method consists essentially of three fundamental changes in the usual cystine assay: 1) preparation of "lanthionized" casein for use in an adequate basal medium; 2) autoclaving the basal medium at 15 lbs. pressure for 15 minutes before preparing the tubes for assay; 3) use of a 2-hour hydrolysis of samples to be assayed to assure minimum destruction of cystine.

[1] Manuscript received March 11, 1955. Contribution from Human Nutrition Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.

TABLE I

TITRATION VALUES FOR TYPICAL STANDARD CYSTINE CURVE

AMOUNT L-CYSTINE PER TUBE	TITRATION WITH 0.05N NaOH	AMOUNT L-CYSTINE PER TUBE	TITRATION WITH 0.05N NaOH
γ	*ml.*	γ	*ml.*
0	1.6	12	9.3
2	4.0	14	9.9
4	6.0	16	10.3
6	7.1	20	11.5
8	7.9	50	14.6
10	8.6		

Preparation of "Lanthionized" Casein. Four grams of casein (16% N) or its equivalent are boiled 20 minutes with 100 ml. of 2% sodium carbonate solution in a 500-ml. flask provided with a reflux condenser. After boiling 1 minute the casein is all in solution. After 20 minutes' boiling, the condenser is removed, the solution cooled, and approximately 4 ml. concentrated hydrochloric acid are added cautiously drop by drop (to avoid excessive foaming) until all carbon dioxide is evolved and the casein is precipitated. Now 100 ml. concentrated hydrochloric are added slowly until the solution contains 20% hydrochloric acid and all the casein is back in solution. The condenser is reconnected and hydrolysis continued in the usual manner for 24 hours. The excess hydrochloric acid is evaporated off on an air bath, the solution adjusted to pH 4.0, filtered and made up to 500 ml. for a stock solution.

Composition of a double-strength basal medium is as follows:

Lanthionized casein hydrolysate	250 ml.
DL tryptophan	400 mg.
L-arginine HCl[a]	320 mg.
Adenine, guanine, and uracil (each)	100 mg.
Salts A[b]	10 ml.
Salts B[b]	10 ml.
Glucose	20 gm.
Sodium acetate	12 gm.

Vitamins in concentrations given in reference 16, except folic acid, which is 0.20 mg. Solution brought to 1,000 ml. volume pH 6.8, autoclaved, and readjusted to pH 6.8 before use.

[a] *L. mesenteroides* P-60 requires large amounts of arginine (see reference 16).
[b] See reference 16.

Leuconostoc mesenteroides P-60 was used as the test organism. The assay procedure was that described in a previous publication (16).

Titration values for a typical standard curve are given in Table I. These values were repeatedly reproduced using numerous preparations of "lanthionized" casein. The humin, filtered from 2-hour hydrolysates, does not affect the titration values (14, 15).

TABLE II

EFFECT OF HYDROLYSIS TIME ON APPARENT CYSTINE CONTENT[a]

TIME	COCONUT GLOBULIN	WHOLE WHEAT	FEATHER MEAL[b]	BLOOD MEAL[c]	α PROTEIN OF SOYBEAN[c]	MALTED BARLEY[c]
Hours	%	%	%	%	%	%
1	1.63	2.25	4.90	1.52	0.24	2.10
2	1.83	2.25	4.70	1.63	0.26	2.02
4	1.53	1.96	3.87	1.55	0.26	1.64
8	1.39	1.54	3.49	1.35	0.23	1.37
24	1.05	1.02	2.82	1.06	0.22	0.96

[a] Calculated to 16% nitrogen, except coconut globulin which was 17.4% N.
[b] Obtained through the courtesy of Charles A. Denton, Animal and Poultry Research Branch, A.R.S., Department of Agriculture.
[c] Obtained through the courtesy of Dr. Harold H. Williams, Department of Biochemistry and Nutrition, Cornell University, Ithaca, N. Y.

Effect of Hydrolysis on Destruction of Cystine. The fate of cystine on prolonged acid hydrolysis was studied. The hydrolysis procedure used in this laboratory is: an amount of food equivalent to 100–200 mg. of protein is hydrolyzed by reflux with 35 ml. of 20% hydrochloric acid. The mixture is evaporated to approximately 7 ml. on an air bath, and washed into a beaker with 200 ml. of distilled water. The solution is adjusted to pH 4.0 with 5 *N* sodium hydroxide, filtered through a fritted glass funnel (14, 15), and washed with water adjusted to pH 4.0. (Caution: filtrate should be at pH 4.0, or if not, it should be made to pH 4.0 and refiltered.) The filtrate is then adjusted to pH 6.8 and made up to 1 liter for assay.

The results obtained on foods containing various amounts of carbohydrate, hydrolyzed for various time intervals are given in Table II, in which 2-hour hydrolysis shows the maximum amount of cystine. It was thought at first that the higher values given by a 2-hour hydrolysate might be due to peptides of cystine with greater activity than the free cystine. It is conceivable that if cystine were free after a 2-hour hydrolysis, other amino acids would also be liberated. Therefore, a pure protein (coconut globulin) and a whole wheat were hydrolyzed for different lengths of time and assayed for eleven amino acids. The results, given in Table III, show that approximately 90% of all the amino acids are either free or in a peptide form small enough to be assayed after 1-hour hydrolysis; that there is no destruction of amino acids on prolonged acid hydrolysis except for cystine; that 8-hour hydrolysis can be used to assay all the amino acids except cystine, for which a 2-hour hydrolysate is recommended; and that the presence of over 80% carbohydrate in the wheat sample does not destroy tyrosine. The observation concerning tyrosine will be discussed in a later paper dealing with the assay of tyrosine.

TABLE III

AMINO ACID VALUES FOR COCONUT GLOBULIN AND WHOLE WHEAT AFTER DIFFERENT PERIODS OF ACID HYDROLYSIS

TIME	ARGININE	HISTIDINE	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYL-ALANINE	THREONINE	VALINE	TYROSINE	CYSTINE
Hours	%	%	%	%	%	%	%	%	%	%	%
	Coconut globulin[a]										
1	15.79	2.12	4.66	6.55	3.20	1.94	4.76	3.12	6.01	2.22	1.63
2	16.02	2.20	4.92	6.71	3.45	1.97	5.01	3.15	6.36	2.61	1.83
4	16.02	2.21	4.73	6.77	3.45	2.00	5.02	3.24	6.36	2.64	1.53
8	16.02	2.30	4.83	7.02	3.60	2.06	5.08	3.21	6.36	2.64	1.39
24	16.02	2.21	4.75	6.81	3.54	1.94	5.05	3.22	6.36	2.64	1.05
Other 24-hour Values	1607	2.20	4.73	6.75	3.80	2.10	5.20	3.06	6.28	2.71	1.20 1.22 1.15
	Whole wheat[b]										
1	4.17	1.94	4.19	5.15	1.76	1.15	4.44	2.50	3.70	2.47	2.25
2	4.34	2.12	4.40	5.85	1.93	1.30	4.96	2.56	3.81	2.44	2.25
4	4.34	2.26	4.43	6.07	2.33	1.37	5.03	2.86	3.75	2.45	1.96
8	4.34	2.33	4.28	6.19	2.50	1.37	5.18	2.92	4.22	2.44	1.54
24	4.34	2.33	4.23	6.25	2.46	1.40	5.05	2.92	4.22	2.44	1.02
Other 24-hour values	4.78 4.50	2.23 2.28 2.54	4.21	6.12 6.34 6.22	2.40 2.31 2.65 2.51	1.28 1.30 1.33 1.32 1.39	4.90 4.92 4.88 5.01	2.86 2.98	4.34 4.13	2.25 2.51 2.35	1.10 1.20 1.18 1.17

[a] Uncorrected for moisture and ash.
[b] Calculated to 16% nitrogen.

TABLE IV

CYSTINE CONTENT OF SOME PROTEINS AND FOODS DETERMINED AT DIFFERENT ASSAY LEVELS

Assay Level	Glycinin		Peanut Globulin		Whole Dried Egg		Hamburger		Pork		Whole Oats		Whole Rye		Navy Bean	
γ	γ	%	γ	%	γ	%	γ	%	γ	%	γ	%	γ	%	γ	%
200	3.16	1.58	3.48	1.74												
400	6.16	1.54	7.40	1.85	5.40	1.35										
600	9.06	1.51	11.10	1.85												
800	12.56	1.57	14.48	1.81	10.48	1.31										
1000							2.42	0.242	2.92	0.292	3.70	0.370				
1200					15.96	1.33										
1600					21.76	1.36										
2000							5.26	0.263	5.66	0.283	7.76	0.388	5.10	0.255	4.56	0.228
3000							7.59	0.253	8.76	0.292	11.49	0.383				
4000							10.32	0.258	11.40	0.285	15.12	0.378	10.60	0.265	9.20	0.230
6000													16.02	0.267	14.52	0.242
8000													21.60	0.270	17.60	0.220
Average		1.55		1.81		1.34		0.254		0.288		0.380		0.264		0.230

Results and Discussion

It is conceded that in a 2-hour hydrolysate only about half of the amino acids are free in the solution. Therefore, the question arises whether the cystine in peptide form reacts differently from the free cystine and hence gives incorrect values. In a study of the reproducibility of cystine values, evidence was found to substantiate the belief that 2-hour hydrolysis gives a truer measure of the cystine content than a longer period.

Reproducibility of Cystine Values. Table IV gives values for cystine at different assay levels and for different products. There is no drift in values at increasing levels. There was a pronounced tendency for high blanks and for levels to drift when the medium was not autoclaved before assay tubes were prepared. This extra autoclaving seems to exert a stabilizing influence on the basal medium and is now being used in this laboratory for all amino acid assays.

To study the effect of peptides of various proteins on cystine recovery, free cystine was added to 2-hour hydrolysates of corn, lima beans, soybean, and wheat. The recoveries of added cystine varied from 97% to 101%, indicating that the peptides present had no effect on the standard curve. A 2-hour hydrolysate of lanthionized casein, to which was added free cystine after hydrolysis, showed no factors present to interfere with recovery of cystine. Not even a mixture of a 2-hour hydrolysate of soybean and a 2-hour hydrolysate of oats influenced the recovery of cystine added *after* hydrolysis.

TABLE V

RECOVERY OF CYSTINE WHEN ONE GRAM OF WHEAT IS HYDROLYZED WITH 20 MILLIGRAMS OF L-CYSTINE FOR 2 AND 24 HOURS RESPECTIVELY

2-Hour Hydrolysis					24-Hour Hydrolysis				
Cystine Added as Wheat	Free Cystine Added	Total Cystine Added	Cystine Found	Recovery	Cystine Added as Wheat	Free Cystine Added	Total Cystine Added	Cystine Found	Recovery
γ	γ	γ	γ	%	γ	γ	γ	γ	%
0.33	2.01	2.34	2.48	106.0	0.20	1.99	2.19	1.55	70.8
0.65	4.02	4.67	4.63	99.0	0.40	3.98	4.38	3.10	70.8
0.98	6.03	7.01	6.90	98.0	0.59	5.97	6.56	4.40	67.0
1.31	8.04	9.35	9.25	99.0	0.79	7.96	8.75	5.95	68.0
1.64	10.05	11.69	11.64	99.6	0.99	9.95	10.94	7.50	68.6
1.96	12.06	14.02	14.00	100.0	1.19	11.94	13.13	8.90	67.8
2.29	14.07	16.36	16.40	100.0	1.39	13.93	15.32	10.50	68.5
2.62	16.08	18.70	18.50	99.0	1.58	15.92	17.50	11.85	67.7
2.94	18.09	21.03	20.50	97.5	1.78	17.91	19.69	13.40	68.0
3.27	20.10	23.37	23.50	100.6	1.98	19.90	21.88	15.00	68.6
Average				99.9					68.6

When cystine is added *before* hydrolysis an entirely different picture is presented. Table V shows recoveries of cystine added to wheat and then hydrolyzed for 2-hour and 24-hour periods. In calculating recoveries of cystine, values obtained on a 2-hour and a 24-hour hydrolysate of wheat were used. Any losses shown would, therefore, be due to loss of the added free cystine. This experiment shows that the lower values obtained on 24-hour hydrolysates are not due to hydrolysis of an active peptide present in a 2-hour digest but to an actual destruction or inactivation of free cystine. The recovery of all the added cystine after 2-hour hydrolysis indicates there is no loss of cystine in any 2-hour hydrolysate, although only about 50% of the cystine is free at that time.

Comparison of the 2-hour and 24-hour cystine values for a series of foods shows that the 24-hour value approaches one-half the 2-hour value; therefore, it is possible that prolonged hydrolysis racemizes the cystine and does not destroy it. That racemization may take place is indicated by the higher values obtained by some colorimetric procedures that do not distinguish between the D and L isomers but depend only on the cystine being free in the solution. For example, values obtained for lactalbumin by the Sullivan colorimetric method for cystine are between 3.5% and 4.1% after hydrolysis for 6 to 8 hours, which compares with our 2-hour value of 4.03% but not our 24-hour value of 2.23%.

Table VI shows the reproducibility of cystine values when determined by separate assay. Each assay was made on a different hydroly-

TABLE VI

REPRODUCIBILITY AND STANDARD DEVIATION OF CYSTINE CONTENT BY SEPARATE ASSAY[a]

Food	Assay 1	Assay 2	Assay 3	Average	S. D.	C. V.
	%	%	%	%		%
Beans, lima	1.12	1.12	1.26	1.17	±0.080	6.9
Casein	0.38	0.37	0.39	0.38	±0.010	2.6
Cheese, Swiss	0.52	0.49	0.50	0.50	±0.016	3.2
Corn, whole	2.38	2.41	2.61	2.47	±0.125	5.0
Lactalbumin	3.95	4.19	3.94	4.03	±0.141	3.5
Milk, dried, skim	0.94	0.97	0.96	0.96	±0.016	1.6
Oats, whole	3.01	2.94	3.01	2.99	±0.040	1.3
Peanut globulin	2.12	1.98	1.93	2.01	±0.098	4.9
Soybean	1.81	1.87	1.96	1.88	±0.080	4.3
Wheat, whole	2.20	2.30	2.25	2.25	±0.050	2.2
Average						3.6

[a] Calculated to 16% N.

sate with a fresh basal medium. Included in this table is the standard deviation and coefficient of variation for representative foods. The average deviation from the mean for all kinds of foods is 3.6%.

It is admitted that in a 2-hour hydrolysate only about 40–50% of the amino acid is free in solution, and it is conceivable that the peptide cystine may react differently. Evidence for ten other amino acids (Table III) indicates that peptides of these amino acids in 2-hour hydrolysates exert no effect, since the 2-hour value agrees with the value obtained on a 24-hour hydrolysate. It is reasonable to assume that cystine peptides react the same way. Recovery experiments indicate no peptide activity on free cystine in 2-hour hydrolysates. The only other way to get high values would be to have a differential reaction between peptides of cystine and free cystine with the glucose (7). This did not occur with the other amino acids in the form of peptides and it is reasonable to assume that the peptides of cystine act no differently. Since it has been shown that free cystine is lost on a 24-hour hydrolysis and not on a 2-hour hydrolysis, and since the recovery of cystine is quantitative after 2-hour and not after 24-hour hydrolysis, a 2-hour hydrolysis period is recommended.

Cystine Contents of Proteins and Foods. The cystine values for some proteins and foods are given in Table VII together with some values found in the literature.

A review of the literature shows that there are wide differences in the cystine values obtained by using various methods, and by different workers using the same method.

Some of these discrepancies may be due to inherent errors in the assay method, others may be due to the method of hydrolysis. In general, our results agree quite well with those obtained by the Baernstein gasometric method (2, 3), but there is no general agreement with the results obtained by use of colorimetric methods. The values obtained on several foods agree with those of Evans (10), who used a differential oxidation method. In the literature there are very few cystine values obtained by use of the microbiological technic. Miller *et al.* (23), using a 2-hour hydrolysis and a microbiological assay, found 2.27% cystine in whole wheat which agrees with our value of 2.25%.

Schweigert *et al.* (29), using a microbiological method and a hydrolysis of meats with 3 *N* hydrochloric for 16 hours at 15 lbs. pressure, obtained 1.40%, 1.30%, 1.30% cystine for beef, lamb, and pork, respectively. Our values are 1.29%, 1.37%, 1.33%. Alexander *et al.* (1), using 2-hour hydrolysis and a microbiological method, obtained 1.0% cystine for lamb.

TABLE VII

CYSTINE VALUES FOR PROTEINS AND FOODS

MATERIALS	N	VALUE FOUND	VALUES FROM LITERATURE
	%	%	
Proteins			
Arachin	18.30	1.28	1.3 (3); 1.5 (6)
Casein	16.00	0.38	0.4 (9); 0.3 (2)
Coconut globulin	17.42	2.04	1.5 (22)
Conarachin	18.20	3.15	2.9 (6)
Cottonseed globulin	18.00	1.31	1.07 (21)
Edestin	18.60	1.45	1.7 (2)
Gelatin	18.30	0.06	0.1 (9); 0.09 (25); 0.00 (2)
Glycinin	17.30	1.67	1.5 (3); 1.12 (21)
Lactalbumin	15.40	4.03	2.9 (9); 4.0 (3)
Ovalbumin	16.00	1.75	1.6 (2); 1.3 (13); 1.8 (28)
Ox muscle	16.00	1.11	1.55 (21)
Peanut, total globulin	18.00	2.01	
Phaseolin	16.00	0.00	
Wheat bran globulin	17.80	0.93	
Zein	16.00	1.40	1.1 (27); 0.85 (21)
Foods[a]			
Beans: Garbanzo (chick peas)	3.08	1.42	
Kidney, red	3.96	0.82	1.0 (21)
Lima	3.46	1.17	0.78 (21)
Navy	3.57	1.03	0.59 (21)
Pinto	3.35	0.62	
Soy	6.21	1.88	1.8 (9); 2.0 (10)
Cereals: Barley, whole	1.98	2.00	1.8 (9)
Buckwheat flour	1.78	2.69	
Corn germ	3.93	0.90	1.8 (9)
Corn, whole	1.78	2.47	1.5 (9); 2.3 (10)
Milo, Plainsman	1.53	1.23	
Oats, whole	2.02	2.99	1.8 (9); 3.0 (10)
Rice, brown	1.27	1.74	
Rice, white	1.19	1.84	
Rye, whole	1.75	2.41	
Cereals (cont.)			
Wheat germ	6.50	0.94	1.4 (9)
Wheat, whole	3.25	2.25	1.8 (9); 2.6 (10); 2.27 (23)
Cheeses: Bleu, Danish	3.39	0.51	
Cheddar	3.87	0.41	
Swiss, imported	4.55	0.50	
Velveeta	2.91	0.61	
Cottonseed meal	6.72	1.72	2.2 (9)
Egg, whole, dried	7.12	2.94	2.2 (24)
Lentils	4.48	0.64	0.68 (21)
Meats: Bologna	1.90	1.58	
Chicken, dark	3.29	1.42	
Chicken, light	3.69	1.25	1.3 (4)
Hamburger	2.81	1.29	1.4 (29); 1.3 (4); 1.4 (12)
Lamb, leg	6.18	1.37	1.3 (29); 1.4 (4); 1.0 (1)
Pork, loin	5.80	1.33	1.3 (29); 1.1 (4)
Turkey, dark	3.30	1.51	
Turkey, light	3.23	1.27	
Milk, dried skim	6.57	0.96	1.0 (9)
Nuts: Almonds	3.58	1.68	
Brazil	9.03	3.06	
Cashews	3.16	2.42	
Coconut, defatted	2.64	1.81	
Filberts	2.55	2.46	0.43 (11)
Peanuts, Spanish	4.59	1.92	1.60 (9); 1.9 (26)
Pecans	1.33	1.86	
Walnuts, English	2.71	1.81	
Peas: Black-eye	3.41	0.70	
Green, split	3.78	0.69	
Yeast: dried, brewer's	7.71	0.76	1.0 (5); 0.5 (8)
Baker's	2.53	0.75	1.1 (9); 0.7 (8)

[a] Calculated to 16% N.

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THE CARBOHYDRATES OF THE GRAMINEAE. VI.

The Nature of the Water-Soluble Polysaccharides of the Flour of Wheat (*Triticum vulgare*)[1]

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ABSTRACT

Wheat gum has been extracted from the flour of spring and winter wheat with water and with aqueous ammonium sulfate. While all samples of the gums contained arabinose, xylose, and glucose, those made by the ammonium sulfate method had the highest pentosan content. The gums probably consist of a mixture of two polysaccharides, one composed of arabinose and xylose, and the other of glucose. The close association of the two polysaccharides was revealed by the fact that their separation by fractional precipitation from aqueous solution with methanol could not be accomplished.

Investigations into the carbohydrates of the grain of barley (11), wheat (12, 14, 15), and rye (5) necessitated an exploratory study of the nature of the water-soluble polysaccharides of wheat flour. The early work of O'Sullivan (16) on the isolation and composition of the water-soluble "amylans" of wheat and that of Freeman and Gortner (10) on methods for the isolation of cereal gums, was not extended until relatively recently when Ford and Peat (8) isolated and carried out methylation studies on a polysaccharide extracted from wheat with aqueous ethanol. Still more recently Perlin (19) has investigated the polysaccharides extracted with aqueous acid and Pence *et al.* (18) examined the pentosans in aqueous extracts of wheat flour freed from protein by adsorption on clay ("filtrol"). Evidence advanced by these authors has shown that the polysaccharides so derived were largely composed of D-xylose and L-arabinose, and that they were frequently associated with D-glucose and D-galactose. The water-soluble materials derived from wheat flour have been shown by several authors, Finney (7), Clendenning *et al.* (4), and Baker *et al.* (2), to influence the various technological processes which utilize flour. The purpose of the present study was to compare the effects of several means of isolation upon the amounts and composition of the recovered pentosans from spring wheat and winter wheat flours. Determination of the composition of the polysaccharides was greatly facilitated by using the micromethod of sugar analysis (6) coupled with paper chromatography (7).

[1] Manuscript received October 4, 1955. Paper No. 3464, Scientific Journal Series, Minnesota Agricultural Experiment Station. This paper forms part of a thesis submitted by K. A. Gilles to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Ph.D., October, 1952.

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Materials and Methods

Quantitative Chromatographic Analysis. A 20-mg. sample of the polysaccharide was hydrolyzed on a boiling-water bath for 24 hours in *N*-sulfuric acid in a sealed tube. The hydrolysate was passed successively through the cation resin Amberlite IR 105 and the anion resin Duolite A4; at each stage the resin column was washed with six successive 10-ml. volumes of distilled water. The deionized sugar solution was concentrated *in vacuo* at 50°C. to give a sirupy mixture of sugars which was weighed. The sirup was dissolved in 1 ml. of a 50% aqueous ethanol solution and stored at 0°C. until ready for use. Immediately prior to chromatographing, the sample solution was brought to room temperature. The chromatographic analysis was carried out on Whatman No. 1 filter paper which had been previously washed repeatedly with water to remove water-soluble impurities that interfere with the colorimetric determination, by the phenol-sulfuric acid method (6).

The paper chromatograms were prepared in the following way. A starting line 20 cm. long was drawn on the Whatman No. 1 paper and 5-cm. margins running the length (57 cm.) of the paper were ruled off from each end of this line, leaving a central portion 10 cm. wide. Within this central portion 40 μl. of the aqueous solution of the hydrolysate were placed along the starting line in a narrow band approximately 0.5 cm. in width and 2 cm. from the edge of each margin. A small amount of this mixture was placed on the starting line in both marginal strips. In addition, an artificial mixture of the sugars known to be present from preliminary qualitative tests was also placed on the starting line in the marginal strips. A blank Whatman No. 1 paper, 10 by 57 cm., was also prepared and developed under the identical conditions used with the paper bearing the hydrolysate.

The chromatograms were developed for approximately 40 hours at constant temperature using the descending technic with the upper layer of a mixture of 1-butanol, ethanol, and water (4:1:5, v/v). The papers were removed and dried in the air at room temperature, and the marginal strips were cut off and sprayed with a solution of ammoniacal silver nitrate (17), *p*-anisidine (13) or N,N-dimethyl *p*-amino aniline (3). After 5 minutes they were heated in an oven at 100°C. until the dark spots appeared, indicating the position of the sugars. The chromatogram was reassembled and, with the aid of lines ruled on the central, unsprayed portion of the chromatogram, using the spots on the marginal strips as indicators, sections of the paper were cut from the central portion so as to include each individual sugar. Iden-

tical pieces of paper with respect to position and area were cut out of the control paper blank.

The sugars were eluted from the paper by placing them in a small beaker containing water, usually 10 to 25 ml. depending upon the color intensity of the control spot. The appropriate elution volume may be arrived at solely as a matter of technic perfection. After standing about 30 minutes with occasional agitation, the eluate was filtered through glass wool and pipetted in 2-ml. quantities into colorimeter tubes; the sugar content of the solution was determined by the phenol-sulfuric acid procedure. The pieces of paper cut from the blank chromatogram were treated in the same manner.

The Phenol-Sulfuric Acid Method for Micro Sugar Determinations. A method employing phenol and sulfuric acid which was developed in this laboratory has been discussed by Dubois *et al.* (6). As applied in this work, the method involved the addition of a 2% (for the pentoses) or a 5% (for the hexoses) solution of redistilled phenol in water (1 ml.) and 95% sulfuric acid (5 ml.) added by a fast-flowing pipette into a sugar solution (2 ml.) containing 10 to 60 μg. of the sugar to be tested. After thorough mixing, the mixture was permitted to stand for 30 minutes before a reading was taken in the spectrophotometer. The optimum concentration of the phenol varies with the individual sugar being analyzed. The optimal absorption maximum must be determined for each sugar. The aldo-hexoses may be read at 490 mμ and the aldo-pentoses at 480 mμ. The standard curve for each

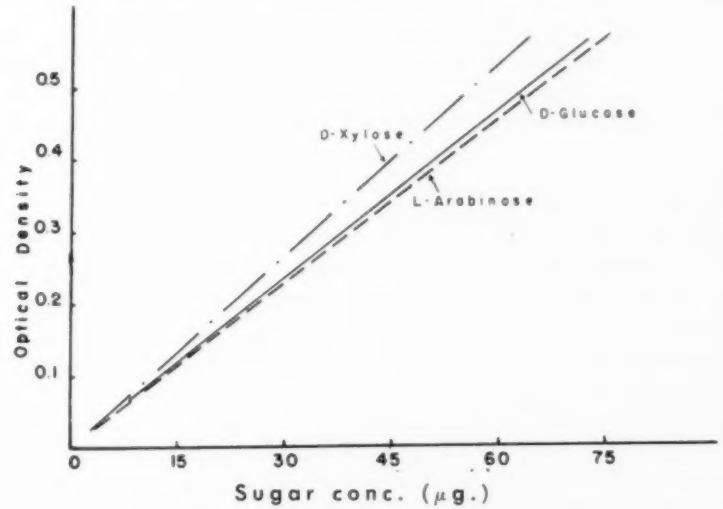

Fig. 1. Representative standard curves.

sugar is established with an authentic sample. A series of representative standard curves is presented in Fig. 1.

Preparation of Wheat Flour Gums. Throughout this communication the term wheat gum will refer solely to the water-soluble polysaccharides, exclusive of starch degradation products, which are derived by the extraction of wheat flour with water.

(a) *Extraction with warm water:* A typical preparation was as follows: Patent flour (50 g.) (protein, 11.7%; ash, 0.47%) from winter wheat and ethanol (300 ml., sp. gr. 0.9) were placed in a flask and agitated in a thermostatically controlled water bath at 42°C. for 3 hours. The residue was collected either by filtration or better still by centrifugation and dried overnight on a Büchner funnel. The residue was extracted with water (600 ml.) at a temperature of 37°C., decanted, and extracted three more times with 250-ml. portions of water. The decanted liquid was centrifuged and concentrated under reduced pressure in a large flask to a volume of about 10 ml. Polysaccharides present in the concentrated solution were precipitated with ethanol (sp. gr. 0.84), filtered, and washed with ethanol first of sp. gr. 0.88 and finally of sp. gr. 0.82. The product was washed with absolute ethanol, anhydrous petroleum ether and dried *in vacuo* over sulfuric acid. The wheat gum was a white amorphous powder – yield, 2.1 g.

(b) *Extraction with cold water:* A sample of commercially milled flour (908 g.) (protein, 13.7%; ash, 0.49%) was stirred overnight with distilled water (2 l.) at an ambient temperature of 2°C. The mixture was allowed to settle and was then centrifuged.

Five distinct layers were discernible in the centrifuge cup. The lower three layers, apparently composed largely of starch, gluten, and sludge, and an upper, foamy layer composed largely of fat and water-soluble proteins, were discarded. When the aqueous extract, approximately 800 ml., was mixed with acetone (2 l.) a precipitate formed. This was collected by centrifugation, dissolved in water (200 ml.), and reprecipitated with acetone (470 ml.). The solvent was removed by air-drying followed by vacuum-drying. The hard, horny product (wt. 19.6 g.) obtained in this way was dissolved in water and precipitated with ethanol (95%). The product was washed successively with ethanol, anhydrous petroleum ether and dried *in vacuo* over sulfuric acid. The yield of the white amorphous light powder was 12.1 g. or 1.3% of the weight of the flour.

(c) *Extraction with ammonium sulfate.* The method employed was a slight modification of the procedure employed by Freeman and Gortner (9, 10), wherein wheat flour was extracted with half-saturated ammonium sulfate in order to avoid contamination of the product

with protein. Two kilograms were dispersed in a half-saturated ammonium sulfate solution (8 l.). The slurry was allowed to stand for several days at room temperature with occasional stirring. After the suspension had settled, the supernatant liquid (6.6 l.) was siphoned off. Solid ammonium sulfate (2.5 kg.) was added with stirring to the supernatant until fully saturated, whereupon precipitation occurred. The precipitate of the original extract was extracted twice with half-saturated ammonium sulfate in an analogous procedure. The precipitates were combined, placed in a cellophane dialysis bag together with toluene and chloroform to prevent microbial growth, and dialyzed until free from sulfate. The solution was then boiled for 0.5 hour with agitation and centrifuged. Into the supernatant liquid (1.9 l.) methanol (7.6 l.) was introduced to effect precipitation of the wheat gum which was centrifuged, washed with methanol, and dried by solvent exchange as outlined above. Yield, (Product A, Fig. 2) 7.1 g. from 2 kg. of flour. The nature of the soluble components of wheat flour extracted by the procedure of Freeman and Gortner was not further elucidated. In our work four fractions were isolated by the manner depicted in Fig. 2.

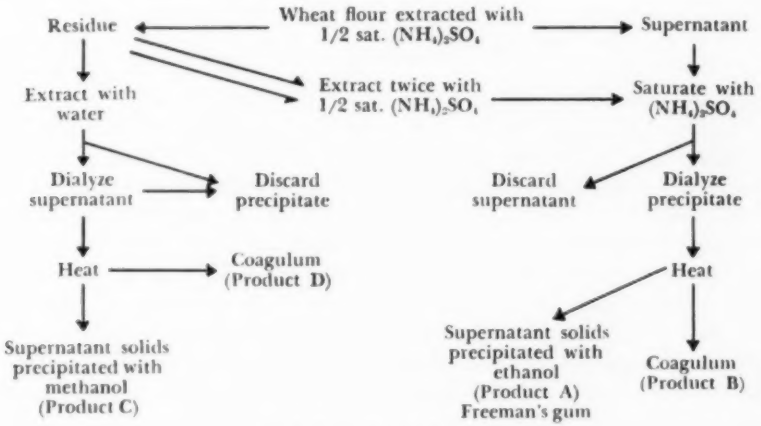

Fig. 2. Preparation of wheat gum.

Fraction A was the gum as prepared essentially by Freeman and Gortner (10) and outlined above. Fraction B was the precipitated residue arising from the heat treatment of Fraction A which yielded 1.87 g. Fractions C and D arose from the water extract of the original flour which had been previously extracted with three portions of half-saturated ammonium sulfate. The extract was dialyzed to remove sul-

fates, heated to boiling, and centrifuged to give Fraction D (yield, 4.73 g.). Fraction C was precipitated from the supernatant with methanol (yield, 2 g.).

Table I summarizes the typical data concerning yields of crude wheat gum derived from various flours together with the ash and crude protein ($N \times 6.25$) of these flours.

TABLE I

TYPICAL YIELDS OF WHEAT GUM DERIVED FROM VARIOUS FLOURS

TYPE OF WHEAT FLOUR EXTRACTED	ANALYSIS OF FLOUR (DRY BASIS)		METHOD OF EXTRACTION	YIELD OF GUM
	Ash	Protein		
	%	%		%
Spring, red dog	3.51	18.6	Water (37°C.)	4.3
Winter, patent	0.47	11.7	Water (37°C.)	4.2
Spring, patent	0.49	13.8	Water (37°C.)	3.9
Winter, red dog	3.43	17.0	Water (37°C.)	3.2
Winter, patent	0.49	13.7	Water (3° C.)	1.3
Winter, patent	0.49	13.7	Half-saturated ammonium sulfate (26°C.)	0.4

Results and Discussion

Chemical Composition. Preliminary paper-partition chromatographic analysis disclosed that the crude wheat gums were similar in composition. All of the gums contained glucose, arabinose, and xylose as revealed by paper chromatography using phenol saturated with water as the irrigating solvent, the respective R_f values being 0.38, 0.48, and 0.55, all in close agreement with authentic samples tested simultaneously. Typical data derived from the analysis of crude wheat gums which appear to be a mixture of pentosan and hexosan (15) are presented in Table II. Bearing in mind that the sample of winter wheat extracted at 37° was a red dog flour while those extracted at 3°C. with water and at 25° using ammonium sulfate were patent flours, the results in Tables I and II indicate that the method of extraction greatly influences the type of crude gum that is ultimately isolated as evidenced by the lack of similarity in specific rotation, pentosan content, and iodine color of the gums.

The high negative specific rotation of the crude gum from winter wheat $[\alpha]_D^{23}$ −108° in *N*-sodium hydroxide (C, 1%), prepared by the ammonium sulfate procedure was accompanied by a high pentosan value as determined by the thiobarbituric acid method of Bailey (1).

Furthermore, the protein content of the crude gum varies with the method of extraction. The ammonium sulfate procedure gives rise to a gum having the highest crude protein value. This occurs even after an extensive dialysis wherein normal tests for ammonium sulfate are made. Apparently this method fails to extract the polysaccharides selectively and it does not leave the protein as a precipitate, as Freeman had hypothesized (9); but it does, however, produce a gum of high pentosan content (see Table IV).

TABLE II

TYPICAL ANALYTICAL DATA OBTAINED FROM CRUDE WHEAT GUMS EXTRACTED FROM WINTER WHEAT PATENT FLOUR

ANALYTICAL TEST	WINTER WHEAT PATENT EXTRACTED WITH HALF-SATURATED $(NH_4)_2SO_4$ (25°C.)	WINTER WHEAT PATENT EXTRACTED WITH COLD WATER (3°C.)	WINTER WHEAT PATENT EXTRACTED WITH WARM WATER (37°C.)
Crude protein (N × 6.25) (%)	22.7	0.89	0.84
Biuret test	+	−	−
$[\alpha]_D^{23}$ *N*–NaOH; C, 1%	−108°	+10.6°	+80.4°
Pentosan (%)[a]	67.0	19.6	13.6
Iodine color	None	Blue-green	Purple
Component sugars by paper chromatography	Glucose Arabinose Xylose	Glucose Arabinose Xylose	Glucose Arabinose Xylose

[a] The rest of the material is a polyglucosan.

Fractionation of the Gums. In an attempt to obtain a wheat gum of high pentosan content, the crude material was fractionated by precipitation from aqueous solution (4%) with methanol, which was slowly added with stirring. These fractions were collected by centrifugation, dehydrated by the method of solvent exchange, and finally dried *in vacuo*. The yields of these gums are given in Table III. Noticeable differences in yields were evident in those fractions precipitated by small amounts of methanol. Moreover, it was observed that 12% of the gum from the ammonium sulfate procedure was insoluble in water.

Determination of the Composition of the Wheat Gum Fractions. Analysis of the fractions (I'–VI') of wheat gum extracted with ammonium sulfate by quantitative paper partition chromatography (6) (see Table IV) shows that the pentose content for all fractions except the second varied between 83% and 94%. Furthermore, the greatest negative specific rotation that was observed for the fractions, prior to hydrolysis, was $[\alpha]_D^{23}$ −111°. This represents a very small change from

TABLE III

FRACTIONAL PRECIPITATION OF WHEAT GUM FROM AQUEOUS SOLUTION WITH METHANOL
(4 g. gum in 100 ml. water)

Methanol Added	Wheat Gum Prepared by Extraction with Water		Wheat Gum Prepared by Extraction with Ammonium Sulfate	
	Fraction	Precipitate	Fraction	Precipitate
ml.		%		%
0	...	...	I′	12.3
25	I	17.4	II′	2.0
50	II	4.0	III′	1.3
75	III	5.6	IV′	0.8
125	IV	5.6	V′	30.5
Excess	V	56.8	VI′	52.7

the initial value of $[\alpha]_D^{23}$ −108° observed in the unfractionated gum.

It is apparent from this that separation of pentosan and hexosan components of the wheat polysaccharide could not be accomplished under these conditions by direct fractional precipitation of the crude mixture. This observation indicates that the effectiveness of the precipitation technic frequently employed as a test for homogeneity of complex polysaccharides is open to question in the case of the wheat gum fractionations.

TABLE IV

CHROMATOGRAPHIC ANALYSIS OF THE HYDROLYZED FRACTIONS OF THE WATER-SOLUBLE POLYSACCHARIDE EXTRACTED FROM WHEAT FLOUR WITH HALF-SATURATED AMMONIUM SULFATE
(4 g. polysaccharide dissolved in 100 ml. water)

Fraction	Methanol Added to Give Precipitate	Mole Ratio of Sugars Present			Pentosan Content
		Glucose	Arabinose	Xylose	
	ml.				%
Crude gum	...	0.17	0.6	1.0	89
I′	Water-insoluble	0.33	1.1	1.0	84
II′	25	0.83	1.4	1.0	71
III′	50	0.25	0.8	1.0	86
IV′	100	0.08	0.5	1.0	94
V′	125	0.33	0.9	1.0	83
VI′	Concentrate of mother liquors	0.33	0.9	1.0	83

Conclusions

The water-soluble polysaccharides derived from wheat flour contain as monosaccharide building units, primarily glucose, arabinose,

and xylose. In reporting the isolation and composition of water-soluble polysaccharides derived from wheat flour, it is imperative that an explicit method of preparation be given, since the nature of the components of the system is greatly dependent upon the means by which these materials are derived from the flour.

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EXTRACTION OF RICE PROTEINS[1]

RUSSELL T. MCINTYRE AND KRISHNA KYMAL

ABSTRACT

The use of an anionic detergent of the alkyl aryl-sulfonate type as an extractant in removing proteins from rice was investigated. Extraction of proteins was most efficient when sodium bisulfite and sodium carbonate were added to an aqueous solution of the detergent. The fineness of sample grinding also increased the extraction efficiency.

When the rice was ground to 100-mesh size, nearly complete dispersal of the nitrogen was accomplished by extractions of 2, 4, and 9 hours' duration employing a solution of 4% Santomerse No. 3, 2% sodium carbonate, and 0.2% sodium bisulfite. Decreasing the detergent concentration to 1% slightly lowered the extraction efficiency.

The common method of extracting rice proteins employing a series of extractions with water, 5% sodium chloride, 70% alcohol, and 0.2% sodium hydroxide leaves about 25% of the protein undispersed. The majority of this protein can be dissolved by use of a detergent solution.

Although rice is the most extensively grown cereal in the world, the literature contains only a small number of reports of investigations concerning the proteins of this grain. Investigations of the rice proteins, as well as many other plant proteins, have been hampered by difficulties encountered in the extraction of these constituents from the starch endosperm. The insoluble nature of these proteins in common solvents systems has been the limiting factor. In one of the earliest studies, Rosenheim and Kajiura (5) reported that the main protein oryzenin belonged to the glutelin class; albumin, globulin, and prolamine types were also present in appreciable quantities. Because of the differences in the solubility types of the proteins, several solutions were employed by previous workers to extract the protein constituents. Complete extraction has not been obtained by the methods reported.

Kik (3) extracted ground rice with 1% sodium chloride, 60% aqueous ethanol, 0.1% sodium hydroxide, and 20% hydrochloric acid, and about three-fourths of the protein was removed. In order to obtain a representative sample of the total protein for amino-acid assay it was necessary to hydrolyze the remaining starch residue and add it to the hydrolyzed extracts. Sturgis, Miears, and Walker (6) used a similar method to separate the proteins in an attempt to determine quantitatively the classical protein types. Extractions with water, 5% sodium chloride, 70% alcohol, and 0.2% sodium hydroxide removed an amount between about 55 and 75% of the total nitrogen. The aver-

[1] Manuscript received February 28, 1955. Contribution from the Department of Agricultural Chemistry and Biochemistry, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana.

age was around 60% extraction. Both of these methods are tedious and time-consuming, and require large volumes of extractants. Moreover, the solubilities of the proteins are such that combination of the fractions results in precipitate formation. Investigations employing these extracts are limited. The need for a more efficient extraction method, as well as a homogenous extract, is obvious.

In a review of protein interaction with synthetic detergents, Putnam (4) indicated that combination of an anionic detergent and protein usually results in solubilization of the protein. Solubility is increased primarily when the detergent part of the molecule and the protein have the same charge. Precipitation may occur at certain conditions of pH and protein detergent weight ratio. The strong dispersive ability of the synthetic detergents is indicated by the studies of Jones and Mecham (2), who found that structural proteins of the keratin class (feather, hoof, etc.) were solubilized by an ionic detergent when used in conjunction with a reducing agent such as bisulfite.

The usefulness of detergents in the extraction and analysis of the corn proteins was reported by Foster, Yang, and Yui (1). These proteins, chiefly of the alcohol-soluble type, were partially dispersed by dilute aqueous solutions of a detergent in alkaline buffer. Extraction was nearly quantitative when bisulfite was added to the extractant.

In view of the above investigations, it appeared probable that a homogeneous solution of the proteins of rice could be obtained by extraction of rice meal with a solution of an anionic detergent. Therefore, the following investigations were undertaken.

Materials and Methods

Preparation of Samples. Brown rice of the Rexoro variety, from the 1953 crop grown at the Rice Experiment Station, Crowley, Louisiana, was used in all investigations. The rough rice was stored at 0°C. for about 6 months before the hulls were removed with a McGill Sheller. The hulled rice was ground with a small laboratory Wiley mill. The moisture content of the ground rice was 14%. The nitrogen content was 1.58%, which corresponds to a protein content ($N \times 5.95$) of 9.40% (dry weight basis).

The fat was removed from the ground rice prior to extraction of the proteins. Samples of rice (5.000 g.) were weighed into a 250-ml. glass-stoppered Erlenmeyer flask. Twenty-five milliliters of diethyl ether were added, and the flask was swirled and allowed to stand 30 minutes. After the ether was decanted, the rice was extracted with two more portions of ether. The flask was allowed to stand in the hood overnight to remove the ether.

Extraction Procedure. Nitrogen was extracted at room temperatures as follows: Thirty milliliters of the solvent mixture were added to the flask containing the defatted rice. The flask was stoppered and shaken. A mechanical shaker (280 strokes per minute) was used to ensure thorough agitation during the extraction. After the flask was shaken the desired length of time, the contents were poured into a centrifuge tube and centrifuged at 2500 r.p.m. for 5 minutes. The centrifugate was decanted directly into an 800-ml. Kjeldahl flask. The residue was transferred back to the Erlenmeyer flask with the aid of fresh solvent and the shaking repeated. In work conducted since these investigations were carried out, the entire series of extractions was made in 90-ml. centrifuge tubes with excellent results. This eliminated transferring from one vessel to another, hence reducing chance for loss of sample. Less time was consumed also.

In one series of experiments, extractions were carried out by Kik's method (3). The residue remaining after the various extractions was then extracted with a detergent solution as described later.

Nitrogen Analysis. The nitrogen content of the extracts, residues, and untreated cereal was determined by the macro-Kjeldahl-Gunning method (A.O.A.C.).

Detergents. Santomerse No. 3,[2] an alkyl benzene-sulfonate, was used in most of the experiments.

Results and Discussion

In the first series of extractions, Santomerse No. 3 (1.0%) and 0.2% sodium bisulfite in water were shaken with 5-g. samples of rice ground to 20-mesh and 60-mesh. The solvent was removed after each day's shaking and fresh extractant added. As indicated in Fig. 1, the extraction efficiency was low after the first day in both cases. Since the majority of the rice protein belongs to the glutelin class (5), an increase in pH was anticipated to aid in protein dispersion. When sodium carbonate was added to the extraction mixture to a concentration of 2%, a sharp increase in extraction efficiency was observed. This observation was also made by Foster, Yang, and Yui (1) in their studies of corn proteins. Since the extraction efficiency was greater when the sample was ground to pass through a 60-mesh screen as compared to a 20-mesh sample, this sample was used in several of the subsequent experiments. However, as will be shown later, a further decrease in particle size would increase the effectiveness of extraction.

The sharp increase in the extraction efficiency of the systems following the addition of sodium carbonate prompted further investiga-

[2] Monsanto Chemical Company, St. Louis, Missouri.

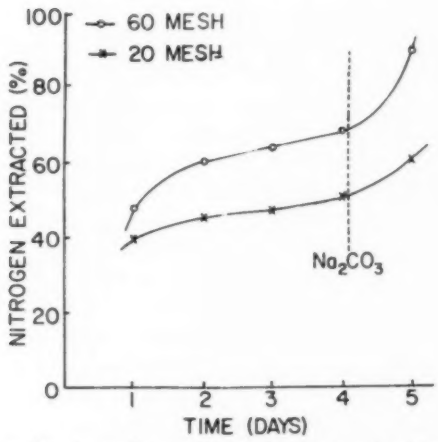

Fig. 1. Effect of particle size on the extraction of rice protein with solutions containing bisulfite and detergent.

tions. It was found that at pH 10 (sodium bicarbonate-sodium carbonate buffer) the extraction was about the same as that which occurred at a higher pH with sodium carbonate alone (pH 11). The results of the extractions described previously indicated that the greater part of the nitrogenous constituents could be removed by several extractions with Santomerse No. 3, sodium bisulfite, and sodium carbonate. In the succeeding experiments, sodium carbonate was employed to maintain the pH at a high level. (Fig. 2.)

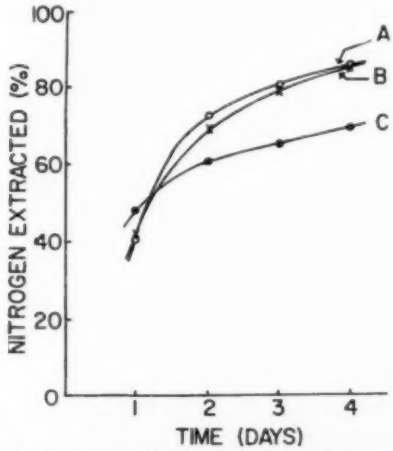

Fig. 2. Extraction of rice protein in buffered and unbuffered solutions. Detergent and bisulfite present. Curve A, carbonate-bicarbonate buffer, pH 10. Curve B, carbonate buffer. Curve C, no buffer.

In preliminary experiments, about as much protein was solubilized during a 2-hour shaking period as during one of 24 hours' duration. The protein solubilized during this stage probably consists of the readily soluble albumin and globulin fractions as well as glutelins from the outer surfaces of the particles. During later stages of extraction, the solvent must penetrate the particles and more time would be needed to remove the protein from the particles. It was therefore assumed that extraction efficiency would not be decreased by shortening the extraction time. A further decrease in particle size was expected to improve the extraction efficiency. Extractions were carried out on samples of two mesh sizes, 60 and 100. The method used in extracting the samples was the same as that previously employed except for modifications of the extraction time. The first extraction was for a period of 2 hours, the second, 4, and the final one, 9 hours. When 60-mesh rice was employed in the extraction, the residue remaining after the 4-hour extraction was ground for 1 minute in a Waring Blendor with a small amount of solvent and transferred back to the extraction flask with the aid of the remaining solvent, and then extracted for 9 hours.

The results of this series of extractions are shown in Fig. 3, curves *A* and *B*. The amount of protein extracted during these shorter extraction periods showed that efficiency was not sacrificed. When the 60-mesh samples were extracted twice more (9 hours for each extraction) the total amount of nitrogen extracted was increased to 95% (data not

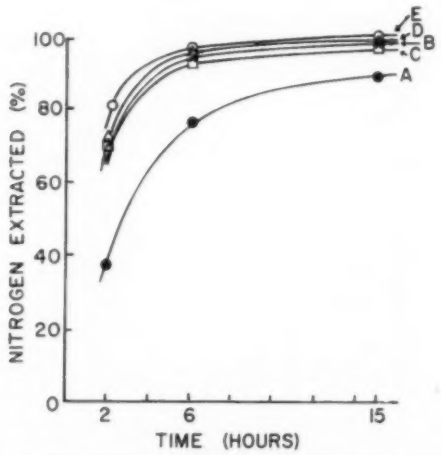

Fig. 3. Extraction of rice proteins with various concentrations of detergent in the presence of sodium carbonate and sodium bisulfite. Curve A, 1% Santomerse No. 3, 60-mesh rice; Curve B, 1% Santomerse No. 3, 100-mesh rice; curve C, 2% Santomerse No. 3, 100-mesh rice; curve D, 3% Santomerse No. 3, 100-mesh rice; curve E, 4% Santomerse No. 3, 100-mesh rice.

shown). The 100-mesh rice showed the greatest extraction efficiency—about 97 to 98%. The small amount of nitrogen extracted during the 9-hour extraction period suggested that further shaking would probably be unprofitable.

The ratio of detergent to rice protein was suggested as possibly affecting the extraction efficiency. Therefore solutions containing 1%, 2%, 3%, and 4% of Santomerse No. 3, as well as 0.2% sodium bisulfite and 2% sodium carbonate, were prepared for a series of extractions employing 100-mesh rice. These concentrations would correspond to a ratio of detergent to protein (wt/wt) of approximately 0.64 to 1, 1.28 to 1, 1.92 to 1, and 2.56 to 1, respectively, for the initial extraction. After the first extraction, this ratio would be much higher since the same amount of detergent is again added after an appreciable quantity (70–80%) of the nitrogen has been removed. The results, shown in curves *B*, *C*, *D*, and *E* of Fig. 3, indicate that extraction was most efficient when the detergent concentration was increased to 4%. The difference between the amounts of nitrogen removed at various concentrations of detergent is small, but nevertheless important when a quantitative extraction is desired. When the volume of the extraction solution was doubled for the first extraction and the normal amounts of solvent used in the final two extractions, the extraction efficiency was about the same as that obtained when extraction was carried out by the usual procedure.

The efficiency of each of the sodium chloride, ethanol, sodium hydroxide, and hydrochloric acid extractions, as employed by Kik (3), is shown in Fig. 4. The total amount of nitrogen extracted by the

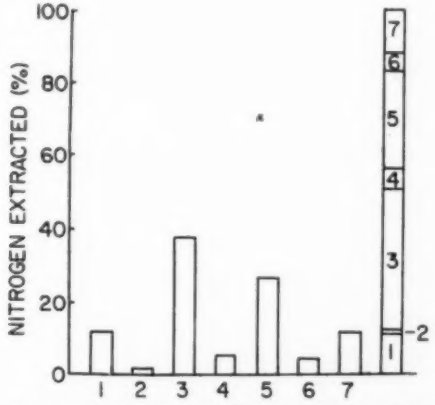

Fig. 4. Extraction of rice protein by method of Kik followed by detergent extraction: 1, 5% sodium chloride; 2, 70% alcohol; 3, 0.2% sodium hydroxide; 4, 20% hydrochloric acid; 5 and 6, 1% Santomerse No. 3, 2% sodium carbonate, and 0.2% sodium bisulfite; 7, residue.

combined extractions was about 60% of the total content. The residue which remained after the hydrochloric acid extraction was shaken overnight with 1% Santomerse No. 3, 0.2% sodium bisulfite, and 2% sodium carbonate. Two of these extractions, each overnight, removed an additional 30% of the total protein. The total amount extracted corresponds to the amount removed with three extractions with the detergent mixture alone (curve *A*, Fig. 3). The Kik method requires several days to complete, whereas the detergent extraction can be accomplished in less than 24 hours.

The use of the detergent method in extracting rice proteins has several distinct advantages. One solution is used throughout, the method is less time-consuming than other procedures, and the extraction is efficient. The presence of the detergent may limit the usefulness of the extracts in some cases. Although the excess detergent is removed slowly by dialysis, it is not known whether the detergent-protein complex may be broken by this method. Another possible way of removing the detergent, which has been applied to other protein-detergent complexes, is to extract it with organic solvents. However, the presence of the detergent would probably not prohibit the use of hydrolysates in chromatographic separation of amino acids by ion exchange methods.

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GRAIN STORAGE STUDIES. XIX.
Influence of Mold Infestation and Temperature on the Deterioration of Wheat During Storage at Approximately 12% Moisture[1]

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ABSTRACT

Surface-disinfected and mold-inoculated samples taken from a lot of sound, hard red spring wheat were stored at 24°C. and 38°C. and approximately 12% moisture after being held at 18% moisture for 7 days at room temperature. After the prestorage treatment the mold count of 31,000/g in the mold-inoculated samples was well within the range of that encountered in many lots of wheat going into commercial storage.

Mold invasion magnified losses in viability and the increases in fat acidity which occurred during temporary storage at 18% moisture.

Upon subsequent storage at 12% moisture, the mold counts of the majority of the samples declined but fat acidity continued to increase at both storage temperatures. Although no significant increase in germ damage was detectable in the samples stored at 24°C. and in the surface-disinfected samples at 38°C., germ damage increased sharply with the mold-inoculated samples stored at 38°C. This clearly demonstrated that greater germ damage can be caused by a combination of high temperature and mold infection than by either factor alone.

Sorger-Domenigg *et al.* (6) recently reported that invasion of wheat by common storage molds during brief temporary storage at moisture contents of 15 to 21% resulted in progressive deterioration of the wheat when stored up to 12 months at moisture contents of 13 and 14% and room temperature. They suggested that these results might help to explain some cases of deterioration in commercial bulks of grain, whose moisture content was supposedly too low for storage molds to grow. However, some of their experimental conditions were more severe than those which are normally encountered in wheat before it reaches terminal storage. Moreover, the moisture content during subsequent long-term storage may have been high enough to permit some invasion by *Aspergillus restrictus*, which has been found recently slowly to invade and kill wheat at moisture contents ranging from 13.5% to 15% (3). The present tests were designed to avoid both of these objections, first by prestorage treatment that would allow a degree of infection by storage molds comparable with that which often occurs in practice, and secondly by subsequent storage of the

[1] Manuscript received July 26, 1955. Contribution from the Departments of Agricultural Biochemistry and Plant Pathology-Agricultural Botany, Institute of Agriculture, University of Minnesota, St. Paul, Minnesota. Paper No. 3388 of the Scientific Journal Series.

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grain at moisture contents low enough to prohibit the continued growth of all known storage molds. In addition, two controlled temperatures of 24°C. and 38°C. were used in storing the grain after the preliminary treatments, since it was suspected that the temperature of the grain during long-term storage might influence the rate of development of germ damage and of biochemical changes that contribute to decreased quality.

Materials and Methods

Wheat. The wheat was of the variety Redman, of a lot having high viability, low fat acidity, and a moderate count of storage molds and with 21% of the seeds yielding storage molds after surface disinfection.

Preliminary Treatment. One lot of 10 kg. was inoculated with a suspension of common storage molds (*Aspergillus glaucus, A. flavus, A. ochraceus, A. candidus,* and *Penicillium*) from moldy wheat, in sufficient water to raise the moisture content of the seeds to 18%. A second lot of 10 kg. was surface-disinfected with a sufficient amount of a solution of 0.1% sodium hypochlorite to raise its moisture content to 18%.

The two lots were stored at 4°C. for 24 hours to allow the moisture to equilibrate. They were then kept for 7 days at 30°C. in stoppered glass containers aerated with sterile air whose relative humidity (85%) was in equilibrium with a grain moisture content of 18%. This treatment was used in order to obtain a degree of infection likely to be found in commercial lots of wheat. After incubation the two lots were air-dried at room temperature until the moisture content of the mold-inoculated lot was approximately 11.5% and that of the surface-disinfected wheat was approximately 12.5%. Each lot was divided into nine subsamples which were placed in screw-top Mason jars. All were stored in the sealed jars at 4°C. for 4 days and mixed frequently to obtain uniform distribution of moisture. The moisture content of each sample was then determined.

Four samples inoculated with molds and four of those surface-disinfected were kept at 24°C. and a similar set of four each were kept at 38°C. Those stored at 24°C. were tested after 2, 4, 8, and 12 months; the samples stored at 38°C. were tested after 1, 2, 4, and 8 months. The ninth sample of each lot was tested immediately after preliminary treatment and the results of these tests were considered to reflect the condition of the other samples at zero time.

Testing Procedures. Each sample when removed from storage was tested for moisture content, fat acidity, viability, germ damage, mold count, deep penetration of the seed by storage molds, milling yield,

flour ash, and baking characteristics. Moisture content was determined by the one-stage, air-oven method specified in *Cereal Laboratory Methods* (1). Fat acidity of ether-soluble material was determined according to the method of Hunter *et al.* (4), viability was determined by the Minnesota State Seed Testing Laboratory, germ damage by the U. S. Grain Inspection Division, Minneapolis, and mold count and deep penetration of the seed by storage molds according to the methods of Christensen (2).

For milling tests, 900 g. of seed were tempered to 15% moisture and ground in a Buhler Laboratory Mill. The flour yield was expressed as percent of total mill products which were recovered as straight grade flour. The ash content of the flour was determined by the rapid magnesium acetate method (1).

For baking tests, 100 g. flour, 3.0 g. yeast, 5.0 g. sugar, 1.0 g. sodium chloride, and an appropriate amount of water were mixed for 3 minutes. The dough was fermented for 2 hours and after proofing was baked at 320°C. for 25 minutes. The loaves were measured by rapeseed displacement 45 minutes after baking and scored the following day. Bromate levels of 0, 1, 2, and 3 mg. of potassium bromate per 100 g. of flour were used with each sample, and the largest volumes were taken as an index of potential baking value irrespective of the bromate levels at which they were obtained.

Results

The major results are summarized in Tables I and II.

The moisture content of the mold-inoculated samples increased by 0.1 to 0.5% in storage, but in no case did it exceed 12.0%. Likewise, the moisture content of the surface-disinfected samples fluctuated somewhat, but the maximum was 12.8%.

Mold Count. The original mold count of 15,000 /g was higher than that usually encountered in the highest grade wheats, but is well within the range of that found in many lots of wheat going into terminal storage (3). The count of 31,000/g in the mold-inoculated samples after prestorage for 7 days at 18% moisture also is by no means excessively high. The mold count of the surface-disinfected samples was originally zero, and remained low throughout the tests. The mold counts of the inoculated samples decreased sharply during the first 2 months of storage and were low at the end of the tests, indicating that the spores originally present were dying. Thus, in the samples inoculated with molds, the mold count was decreasing as the amount of germ damage and other evidences of deterioration were increasing. This indicates, as suggested previously (6), that mold count alone may

not be a reliable index of present or future deterioration unless it is possible to obtain samples when the molds are increasing or at their peak, or at least before the majority of the spores have died.

Deep Penetration of the Seed by Storage Molds. Originally 21% of the seeds, after surface disinfection, yielded molds; this degree of infection is commonly encountered in high-grade wheats (3). After prestorage treatment, the surface-disinfected seeds were free of molds, whereas the mold-inoculated samples were 100% infected. At 24°C. deep penetration of the surface-disinfected seeds remained essentially at zero and that of the mold-inoculated seeds remained essentially at

TABLE I

CHANGES IN SURFACE-DISINFECTED AND MOLD-INOCULATED SAMPLES OF WHEAT CONTAINING APPROXIMATELY 12 PER CENT MOISTURE UPON STORAGE AT 24°C.

WHEAT TREATMENT	MONTHS STORED				
	0	2	4	8	12
	Fat acidity, mg. potassium hydroxide/100 g wheat, dry basis				
Untreated	17.4				
Mold-inoculated	21.0	38.4	43.4	53.4	91.8
Surface-disinfected	20.2	27.2	31.0	38.9	40.9
	Viability, per cent				
Untreated	93				
Mold-inoculated	63	49	60	56	61
Surface-disinfected	93	91	92	92	90
	Germ damage, per cent				
Untreated	0				
Mold-inoculated	2	10	2	5	4
Surface-disinfected	2	trace	4	trace	0
	Mold count, No./g wheat				
Untreated	15,000				
Mold-inoculated	31,000	2,000	8,400	3,600	2,800
Surface-disinfected	0	200	600	0	0
	Deep penetration, per cent of kernels invaded				
Untreated	21				
Mold-inoculated	100	18	86	86	100
Surface-disinfected	0	14	2	0	3
	Moisture content, per cent				
Mold-inoculated	11.7	11.9	11.9	11.8	11.7
Surface-disinfected	12.5	12.7	12.7	12.7	12.4
	Loaf volume, cc.				
Untreated	795				
Mold-inoculated	750	765	780	705	690
Surface-disinfected	760	780	795	765	770

100%. At 38°C. deep penetration of the surface-disinfected seeds remained at zero and that of the mold-inoculated seeds decreased to 8% at the end of 8 months.

Viability. During the prestorage incubation at 18% moisture the viability of the surface-disinfected sample remained at 93%, while that of the sample inoculated with molds decreased to 63%. In subsequent storage for 12 months at 24°C. the viability of both samples remained essentially constant. However, upon storage at 38°C. for 8 months, the viability of both samples decreased, to 30% in the case of the mold-inoculated sample and to 3% in the case of the surface-

TABLE II

CHANGES IN SURFACE-DISINFECTED AND MOLD-INOCULATED SAMPLES OF WHEAT CONTAINING APPROXIMATELY 12 PER CENT MOISTURE UPON STORAGE AT 38°C.

WHEAT TREATMENT	MONTHS STORED				
	0	1	2	4	8
	Fat acidity, mg. potassium hydroxide/100 g wheat, dry basis				
Untreated	17.4				
Mold-inoculated	21.0	36.1	49.6	60.9	79.4
Surface-disinfected	20.2	21.2	31.5	41.8	62.2
	Viability, per cent				
Untreated	93				
Mold-inoculated	63	49	45	47	30
Surface-disinfected	93	93	78	71	3
	Germ damage, per cent				
Untreated	0				
Mold-inoculated	2	3	11	22	27
Surface-disinfected	2	trace	trace	5	5
	Mold count, No./g wheat				
Untreated	15,000				
Mold-inoculated	31,000	1,000	1,000	1,600	0
Surface-disinfected	0	0	1,400	200	0
	Deep penetration, per cent of kernels invaded				
Untreated	21				
Mold-inoculated	100	20	58	14	8
Surface-disinfected	0	0	10	0	0
	Moisture content, per cent				
Mold-inoculated	11.7	11.7	11.7	11.7	11.8
Surface-disinfected	12.5	12.7	12.7	12.5	12.5
	Loaf volume, cc.				
Untreated	795				
Mold-inoculated	750	705	735	770	630
Surface-disinfected	760	750	755	765	660

disinfected sample. Apparently this temperature alone at a moisture content as low as 12% is slowly lethal to wheat.

Germ-Damaged or "Sick" Wheat. There was a slight development of germ damage in both the surface-disinfected and mold-inoculated samples after the prestorage treatment, although it may be doubted whether this is significant, since in three of the four subsequent test periods the germ damage of the surface-disinfected samples ranged from zero to a few kernels. When stored for 12 months at 24°C. there was a possible slight increase in germ damage of the samples originally inoculated with storage molds, but no increase in those that were surface-disinfected. When stored for 8 months at 38°C. the germ damage of the surface-disinfected sample fluctuated from a trace to 5%, while that of the samples originally inoculated with molds increased consistently with time, reaching 27%. This seems rather conclusive evidence that a preliminary invasion of the seed by storage molds, of a degree often encountered in commercial lots of wheat, may result in really significant increases in "sick" wheat with time, if the seeds are stored at 38°C. In other words, a combination of original mold invasion and a high storage temperature will result in much greater germ damage than will high temperature alone.

Fat Acidity. During the prestorage period of 7 days, the fat acidity of the mold-inoculated and surface-disinfected samples increased only slightly. In subsequent storage at 24°C. the fat acidity of the samples inoculated with molds increased much more rapidly than that of the surface-disinfected samples. At the end of 12 months, the fat acidity of the mold-inoculated sample had risen to four and one-half times its original value, whereas the fat acidity of the surface-disinfected sample had only doubled. With the samples stored at 38°C. the fat acidity of both the mold-inoculated and surface-disinfected samples increased more rapidly than at 24°C. Again the fat acidities of the mold-inoculated samples were considerably higher than those of the surface-disinfected samples. At both temperatures the evidence shows that a preliminary invasion of the seed by storage molds leads to subsequent increases in fat acidity even though the seed is stored at a moisture content too low for the molds to continue to grow. Apparently some of the mold-produced lipases continue to act at moisture contents below those that permit mold growth.

Flour Yield and Ash Content. The flour yields and ash contents of the original samples and of those stored for 8 to 12 months at 24°C. and 38°C. remained essentially the same. Apparently deterioration must be rather far advanced before flour yield and ash content are appreciably affected.

Baking Quality. There was no pronounced change in the baking qualities of any of the samples stored at 24°C. Although the loaf volumes of the mold-inoculated samples were consistently lower than those of the surface-disinfected samples, the differences in most cases were too slight to be considered significant. Storage at 38°C. resulted in lower loaf volumes than storage at 24°C., but the loaves obtained from all the stored samples had good internal characteristics.

Discussion

Sorger-Domenigg *et al.* (6) have shown that, once wheat has been invaded by common storage molds, deterioration as measured by increases in fat acidity and germ damage may continue even after the wheat has been dried to 13–14% moisture. The present studies generally confirm these findings. Moreover, evidence of deterioration was found in samples with a mild degree of mold infection such as is likely to occur in commercial practice and at moisture levels as low as 12%. In addition it was clearly demonstrated that greater germ damage could be caused by a combination of high temperature and mold infection than by either factor alone.

In the mold-infected samples stored at 38°C., 20% germ damage appeared in 4 months; in contrast, the infected samples stored at 24°C. showed only a slight amount of germ damage after 12 months' storage. This is in line with the findings of MacDonald and Milner (5), who showed that browning in wheat germ was promoted by elevated temperatures. It is quite possible that molds produce precursors for the browning reaction and that they may continue to produce these precursors even at moisture contents too low for the molds to continue to grow. The browning reaction would then proceed at noticeable rates at adequate temperatures in the originally mold-invaded samples.

The data clearly show the importance of molds in the deterioration of grain stored at moisture contents as low as 12%. Although surface-disinfected wheat stored at 38°C. for 8 months fell in viability to 3% whereas the mold-inoculated sample fell only to 30% viability, all other evidence of deterioration, including germ damage, was considerably higher in the mold-inoculated sample.

Acknowledgments

This investigation was supported by a grant from the Millers' National Federation and the National Grain Trade Council. The authors are indebted to the U. S. Grain Inspection Office, Minneapolis, for determining the extent of germ damage and to the Minnesota State Seed Testing Laboratory for germination tests.

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GRAIN STORAGE STUDIES. XXII.
Influence of Oxygen and Carbon Dioxide Concentrations on Mold Growth and Grain Deterioration[1]

ANNE PETERSON, VERA SCHLEGEL, B. HUMMEL, L. S. CUENDET, W. F. GEDDES, AND C. M. CHRISTENSEN

ABSTRACT

Subsamples of sound hard red spring wheat, 88% viable, conditioned to approximately 18% moisture were stored at 30°C. in a respiration apparatus which allowed suitably humidified gas mixtures of known composition to be passed through the grain at a controlled rate. Two series of gas mixtures were employed. One comprised mixtures of oxygen and nitrogen containing from 0.2 to 21.0% oxygen; the other contained 21% oxygen and varying quantities of carbon dioxide and nitrogen to provide carbon dioxide levels from 0.02 to 79.0% by volume.

Daily respiration trials and analysis of the wheats after 16 days' storage showed that mold growth, germ damage, fat acidity, and respiration rate all gradually decreased as the oxygen concentration was lowered. Some mold growth occurred at 0.2% oxygen but the wheat maintained its viability; a respiratory quotient of 8.8 indicated that anaerobic metabolism was taking place. As the oxygen concentration was increased, viability decreased and the sample aspirated with air was only 7% viable after 16 days.

In the presence of 21% oxygen, increasing levels of carbon dioxide had little effect on respiration rate until the concentration exceeded 13.8 to 18.6%, when a very sharp and marked inhibition of respiration, mold growth, and development of fat acidity occurred. At large carbon dioxide concentrations (50 and 79%), the viability of the wheat remained high and there was little or no germ damage.

In recent years, the possibility of preserving bulk grain by hermetic or air-tight storage has aroused widespread interest (11, 12, 15, 20, 24). The respiratory processes of the grain, with its associated molds (and sometimes insects), tend to deplete the oxygen supply and increase the carbon dioxide content of the interseed atmosphere; under sealed conditions a point would eventually be reached at which mold growth, insect development, and further spoilage of the grain would be inhibited. The question arises whether the depletion of oxygen or the accumulation of carbon dioxide is the more important factor.

Fungi are commonly regarded as being strictly aerobic, although there are several reports in the literature of certain species that will continue to grow, but at a greatly reduced rate, under low oxygen concentrations (10, 13, 19, 23). The investigations of Bottomley *et al.* (3) revealed that nearly anaerobic conditions (0.1% oxygen) failed to pre-

[1] Manuscript received August 26, 1955. Contribution from the Department of Agricultural Biochemistry and Department of Plant Pathology and Agricultural Botany, Institute of Agriculture, University of Minnesota, St. Paul, Minnesota. Paper No. 3401 of the Scientific Journal Series. The data in this paper are taken in part from theses presented to the Graduate School of the University of Minnesota by Vera Schlegel and Anne Peterson, in partial fulfillment of their requirements for the M.S. degree in September 1954 and March 1955 respectively.

vent the growth of all mold species present on corn but greatly reduced their sporulation. In general, carbon dioxide has been shown to inhibit mold growth (4, 5, 6), but several investigators have reported that low concentrations of this gas have a stimulating effect (9, 10, 22). In studying the respiration of soybeans containing 19.1% moisture, Milner and Geddes (18) did not observe any respiratory inhibition until the carbon dioxide content of the interseed atmosphere reached 12 to 14%. Similar studies with wheat (17) indicated that the respiration of this grain at elevated moisture levels was somewhat more sensitive to carbon dioxide than that of soybeans.

The present study was undertaken to determine, independently, the effect of various oxygen and carbon dioxide concentrations upon the growth of molds and on the respiration, viability, fat acidity, and development of germ damage in damp wheat.

Materials and Methods

Subsamples of sound hard red spring wheat (Henry), 88% viable, conditioned to approximately 18% moisture, were stored at 30°C. in an apparatus which allowed suitably humidified gas mixtures of known composition to be passed through the grain at a controlled rate. Two series of gas mixtures were employed; one comprised mixtures of oxygen and nitrogen containing from 0.2 to 21.0% oxygen by volume, and the other contained 21% oxygen by volume and varying quantities of carbon dioxide and nitrogen to provide carbon dioxide levels from 0.02 to 79.0% by volume.

Apparatus for Determination of Respiratory Rate. At first, the apparatus constructed by Milner and Geddes (18) was used, which provides for the simultaneous measurement of oxygen consumption and carbon dioxide production under conditions of continuous aeration. Air is drawn through a humidifying solution, a sample of grain, and a spirometer respectively by means of leveling bulbs connected to the spirometer containing a solution of saturated calcium chloride. The respired gaseous mixture is then analyzed for carbon dioxide and oxygen content with the Haldane-Henderson apparatus. For the present study the various gas mixtures were purchased in cylinders, and difficulties were encountered in regulating the gas flow through the grain. Also, a saturated solution of calcium chloride was unsuitable for use in the spirometers, as carbon dioxide, in particular, was appreciably soluble in this solution and thus caused a change in the composition of the gases employed. In addition, significant amounts of carbon dioxide were lost through the rubber-tubing connections. These effects were particularly noticeable with gas mixtures containing high

percentages of carbon dioxide. It was therefore necessary to construct a new apparatus which would not have the limitations described above.

A diagrammatic sketch of the apparatus is shown in Fig. 1.

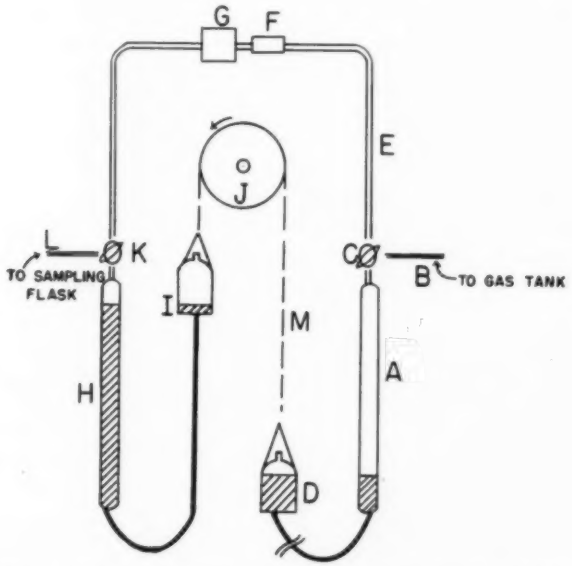

Fig. 1. Diagrammatic sketch of apparatus used to determine the respiratory rate of wheat at various oxygen and carbon dioxide concentrations. *A*, spirometer for gas supply; *B* and *C*, gas line and stopcock to connect system to gas supply; *D* and *I*, leveling bulbs; *E*, gas line; *F*, humidifying solution; *G*, respirometer vessel containing wheat; *H*, spirometer for receiving gas from respirometer; *K* and *L*, stopcock and gas line for withdrawing gas sample.

In order to pass the gaseous mixtures at a uniform rate and constant pressure through the wheat, two leveling bulbs suspended on the same cogwheel and two spirometers were used for each sample of seed tested. Mercury was used in the spirometers and leveling bulbs, and because of its high density the dimensions of the apparatus had to be considerably reduced. The volume of each spirometer was scaled down from 2000 ml. to 250 ml. and the weight of seed tested from 185 g. to 25 g. To suspend the heavy leveling bulbs, bicycle chains and cogwheels were used.

Most of the rubber tubing was replaced by capillary glass tubing, and where flexible connections were essential tygon tubing was used as it is practically impermeable to carbon dioxide, oxygen, and nitrogen. The respirometer vessels were designed with ground-glass joints to eliminate the danger of gas leakage. These containers were also pro-

vided with mercury traps (Fig. 2), so that the mercury of the receiving spirometers (*H*, Fig. 1) could never get in contact with the seeds. The humidifying solution (5 ml. of 22% sulfuric acid) to maintain 18% moisture was placed in a container, *G*, designed to prevent any loss of gas. The respirometer vessel and container for the humidifying solution are shown in Fig. 2.

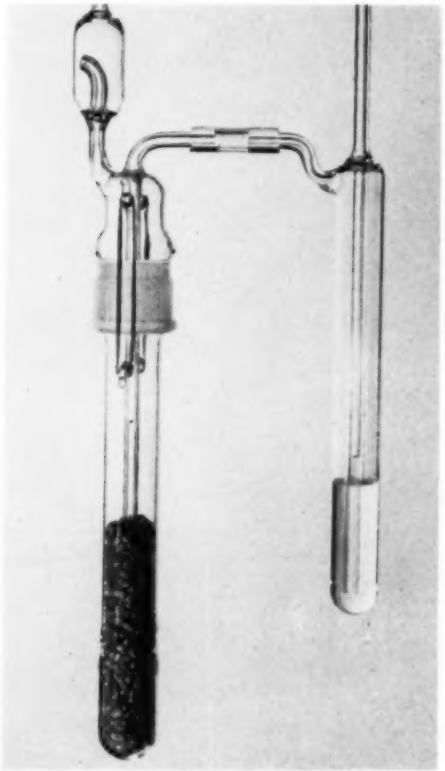

Fig. 2. Respirometer with mercury trap and container for the humidifying solution.

The principle of operation is as follows: A sample of the gas mixture is drawn into spirometer *A*, through side tube *B* and stopcock *C*, by lowering the leveling bulb *D*. The stopcock *C* is then turned so as to connect the spirometer *A* with line *E*, and the gas mixture is passed through the seed in the respirometer vessel *G*. The humidifying solution, as well as the seed sample, are maintained at constant temperature (30°C.) in a thermostatically controlled water bath. A uniform

rate of gas flow through the humidifying solution *F* and the seed sample is maintained by gradual ascent of mercury in spirometer *A* and corresponding mercury descent in spirometer *H*. The gas mixture is moved regularly from spirometers *A* and *H* by the slow ascent and descent of straight-sided leveling bulbs *D* and *I*, fastened by set-screws to a lineshaft which is driven at constant speed by a synchronous motor. The number of revolutions per hour of the cogwheel can be adjusted so that from 100 to 1000 ml. of gas can be passed through the seed in 24 hours. When sufficient respired gas is accumulated in spirometer *H*, a sample can be withdrawn for analysis through stopcock *K* and line *L* by raising leveling bulb *I*. To begin the next trial the balance of the gas in spirometer *H* is discharged to the atmosphere and spirometer *A* is filled with a new charge of the gas mixture.

The lineshaft served to control six individual respirometer systems, thus permitting the simultaneous testing of six seed samples in one constant-temperature bath.

Analytical Methods. Samples of respired gas were collected daily for 14 to 20 days and analyzed for oxygen and carbon dioxide. All samples containing less than 30% of carbon dioxide by volume were analyzed in a Haldane-Henderson apparatus according to the method of Peters and Van Slyke (21); those containing higher levels were analyzed in the Van Slyke apparatus. The results were expressed as mg. of carbon dioxide produced by 100 g. of dry seed in 24 hours. The average respiratory quotients were also computed.

Moisture contents were obtained by the two-stage air-oven method (1).

Viability of the kernels before and after the respiration trials was determined by incubating 100 seeds in Petri dishes between sheets of wet filter paper at room temperature and counting the number of kernels which had normal sprouts in 5 to 7 days.

Germ damage was determined by the Grain Inspection Division, U. S. Marketing Service, Minneapolis.

Mold counts were made according to Christensen (8) by grinding the seed in a Wiley mill and culturing the resultant meal in malt-salt-agar for 5 to 7 days at room temperature (20°–25°C.).

To determine the percentage of seeds in which internal molds were present, 50 seeds were washed in running tap water, then surface-disinfected by washing in a 1.5% solution of sodium hypochlorite for 2 minutes (to eliminate any contaminants acquired from the tap water or from the air), placed on agar in Petri dishes, and incubated at room temperature. Colonies were counted after 5 to 7 days. Moisture determinations were made by the two-stage air-oven method (1).

Fat acidity was determined on the lipids obtained from 3.0 g. of the ground wheat (micro Wiley mill, No. 20 sieve) by successive extractions with absolute ethanol and diethyl ether for 4 hours. The extracts were combined and the solvent evaporated in a nitrogen atmosphere under reduced pressure. The residual oil was dried and the free fatty acids determined according to the method of Ames and Licata (2), as modified by Hunter *et al.* (14). The lipids were dissolved in a mixture of benzene and isopropanol (1 + 1 by volume) and titrated with 0.01 *N* potassium hydroxide solution in isopropanol. The free fatty acids were expressed as mg. of potassium hydroxide per 100 g. of wheat on a 14% moisture basis.

Results

Effects of Oxygen Concentration. The mold counts for different species of molds found in the wheat after it was exposed to various oxygen concentrations and zero carbon dioxide concentration for 16 days are recorded in Table I. The sample exposed to air (20.6% oxygen) had the highest mold count and the mold population decreased gradually as the oxygen concentration was lowered. However, there was significant mold growth even in the sample aspirated with tank nitrogen (0.2% oxygen). The data indicate that *Aspergillus glaucus* will survive at lower oxygen concentrations than *Penicillium* and *A. flavus*.

The viability of the wheat aspirated with 0.2% oxygen was 86% after storage for 16 days at about 18% moisture, but the presence of

TABLE I

Effect of Oxygen Concentration on Mold Population and Distribution in Wheat Stored 16 Days at 18 Percent Moisture and 30°C.

Oxygen	Mold Assay	
	Mold Count	Mold Species
%	*molds/g*	
0.2	7,000	*Aspergillus glaucus*
2.3	192,000	*Penicillium*
	288,000	*A. glaucus*
4.3	100,000	*Penicillium* sp.
	800,000	*A. glaucus*
	100,000	*A. flavus*
8.0	100,000	*Penicillium*
	560,000	*A. glaucus*
20.6	680,000	*Penicillium*
	390,000	*A. glaucus*
	56,000	*A. flavus*

only 2.4% oxygen was sufficient to lower the viability to 38% (Fig. 3). Additional increases in oxygen concentration produced further decreases in viability and the sample aspirated with air was only 7% viable. Germ damage was directly related to the oxygen concentration (Fig. 3). The wheat exposed to air contained 80% germ-damaged kernels at the end of the trial, whereas no damage could be detected in the sample kept under 0.2% oxygen.

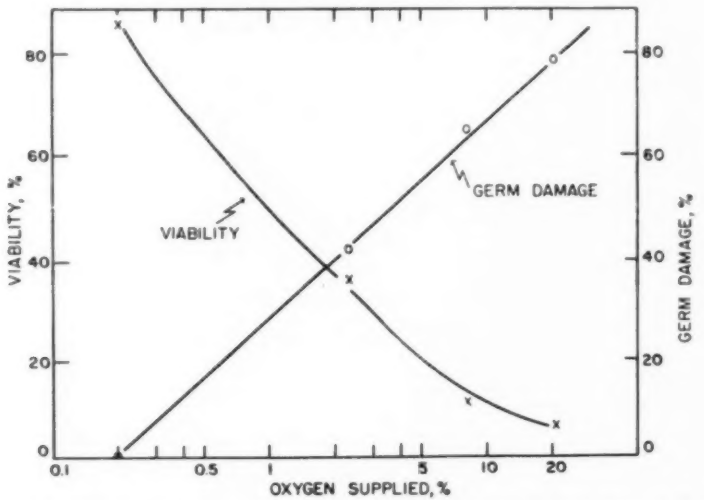

Fig. 3. Effect of oxygen concentration on viability and germ damage of wheat incubated 16 days at 30°C. and 18% moisture. The gas mixtures did not contain carbon dioxide.

The changes in respiratory rate with oxygen concentration (Figs. 4 and 5) paralleled the changes in mold count. Production of carbon dioxide was at a maximum in the sample which respired in air and decreased gradually with reduced oxygen concentration. However, even with only 0.2% oxygen in the gas mixture, respiration was not completely halted. A high respiratory quotient (8.8), indicative of anaerobic metabolism, was observed in this case.

The trends observed in the fat acidity values were essentially the same as for mold population and respiration (Fig. 5). The samples stored under high oxygen concentrations were high in fat acidity and declined gradually with a decrease in oxygen concentration.

Effects of Carbon Dioxide Concentration. The gas mixtures in which the carbon dioxide concentration was varied all contained approximately 21% oxygen. When the carbon dioxide concentration was increased from 0.02 to 12.2% there was a slight increase in mold count

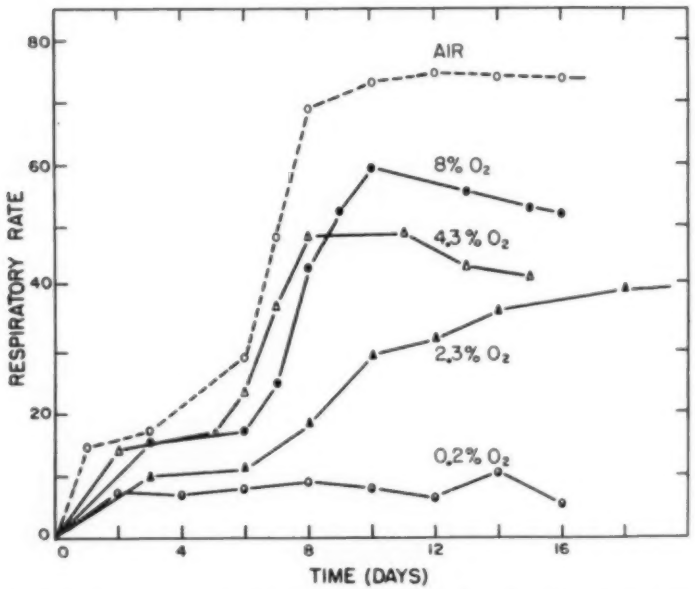

Fig. 4. Effect of oxygen concentration on the respiration rate of wheat incubated at 30°C. and 18% moisture. Respiratory rate is expressed as mg. of carbon dioxide per 100 g. dry matter per 24 hours. The gas mixtures did not contain carbon dioxide.

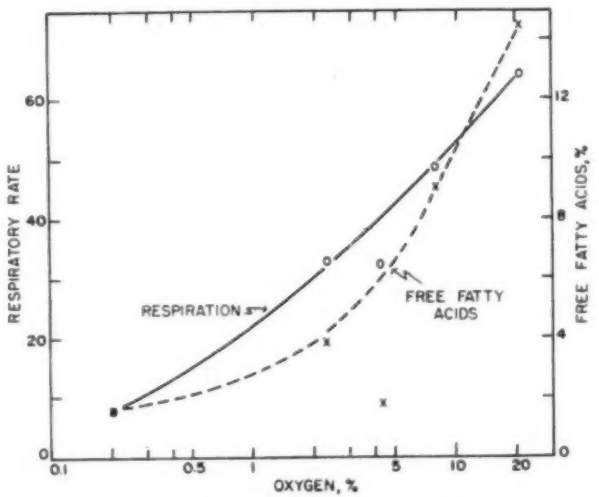

Fig. 5. Effect of oxygen concentration on the mean daily respiration rate and fat acidity of wheat after incubation for 16 days at 30°C. and 18% moisture. Respiration rate is expressed as mg. of carbon dioxide per 100 g. dry matter per 24 hours. Fat acidity is expressed as mg. of potassium hydroxide per 100 g. of wheat on a 14% moisture basis. The gas mixtures did not contain carbon dioxide.

(Fig. 6). When the concentrations were further increased there was a very sharp decrease in mold growth. With 50.0 and 79.0% carbon dioxide, mold growth was almost completely inhibited. The percentage of infested seeds and types of molds present (Table II) indicate that *Aspergillus glaucus* is more resistant to high carbon dioxide concentrations than other molds such as *A. flavus, A. candidus, Alternaria,* and *Penicillium* spp. (Table II).

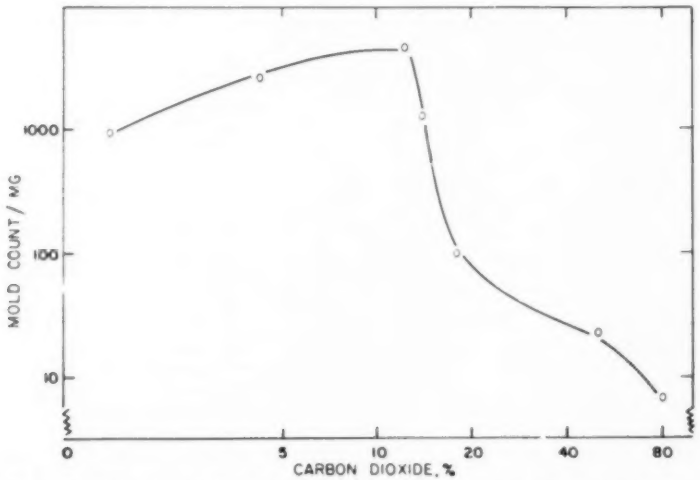

Fig. 6. Effect of carbon dioxide tension on mold count (colonies per mg.) of wheat incubated 20 days at 30°C. and 18% moisture. The gas mixtures contained 21% oxygen.

Increases in carbon dioxide concentration had no apparent influence on viability until the concentration was between 18.5 and 50.0%, when further increases resulted in higher viability (Fig. 7). Germ damage decreased gradually as the carbon dioxide concentration increased. However, the samples stored in 79% carbon dioxide showed no increase in germ damage during incubation.

The effect of carbon dioxide concentration on respiration of the wheat and accompanying molds is shown by daily respiratory rates (Fig. 8) and by the average daily rates over the 18-day trial (Fig. 9). Although a carbon dioxide concentration of 4.3% had a slight accelerating influence on respiration, there was no marked effect at levels below 13.8%. However, when the concentration exceeded 18.6%, the respiratory rate decreased very sharply and there was no measurable respiration in the presence of 50 and 79% carbon dioxide.

The average respiratory quotients decreased from a value of 1.0 for the wheat which respired in air to 0.55 for carbon dioxide con-

TABLE II

EFFECT ON MOLD GROWTH OF STORING WHEAT CONTAINING 18 PERCENT MOISTURE AT 30°C., AT VARIOUS CARBON DIOXIDE CONCENTRATIONS IN THE PRESENCE OF 21 PERCENT OXYGEN[a]

CARBON DIOXIDE CONCENTRATION	INTERNALLY INFESTED KERNELS[a]	MOLD SPECIES
%	%	
0.02	100	*Aspergillus glaucus*
	10	*Penicillium* sp.
	4	*A. flavus*
1.4	86	*A. glaucus*
	24	*Alternaria*
	5	*Penicillium* sp.
4.3	86	*Aspergillus glaucus*
	36	*Alternaria*
	14	*Aspergillus candidus*
	4	*Penicillium* sp.
12.2	97	*Aspergillus glaucus*
	11	*Alternaria*
	7	*Penicillium* sp.
13.8	96	*Aspergillus glaucus*
	14	*Alternaria*
	7	*Penicillium* sp.
	1	*Aspergillus flavus*
18.6	100	*A. glaucus*
	15	*Penicillium* sp.
	8	*Alternaria*
	4	*Aspergillus flavus*
50.0	13	*A. glaucus*
	33	*Alternaria*
79.0	35	*Aspergillus glaucus*

[a] The values show the percentage of kernels infested with each species and hence may total more than 100%.

centrations of 13.8 to 18.6%. The decrease in respiratory quotient when mold growth is inhibited has been noted previously in these laboratories (17).

The trends in fat acidity closely followed the changes in mean daily respiration rate (Fig. 9).

Discussion

Independent changes in the oxygen and carbon dioxide concentrations under which wheat is held at a moisture content and temperature favorable for mold growth had quite marked but different effects on the various properties studied. Decreasing the oxygen concentration in the absence of carbon dioxide progressively retarded mold growth, losses in viability, respiration, development of fat acidity, and germ damage. There was a close relation between mold count and the other

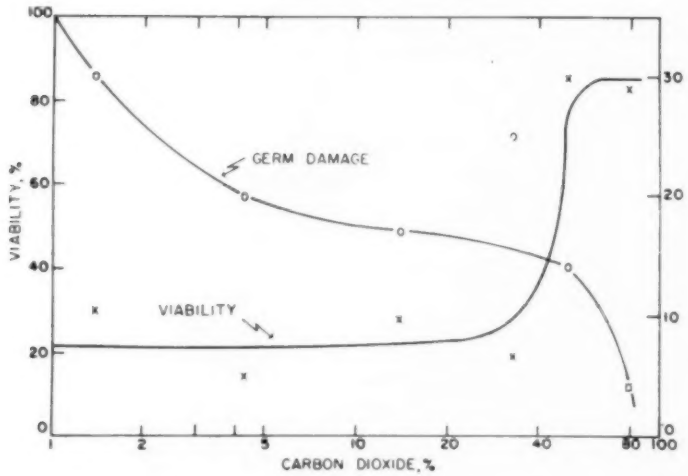

Fig. 7. Effect of carbon dioxide concentration on viability and germ damage of wheat incubated 20 days at 30°C. and 18% moisture. The gas mixtures contained 21% oxygen.

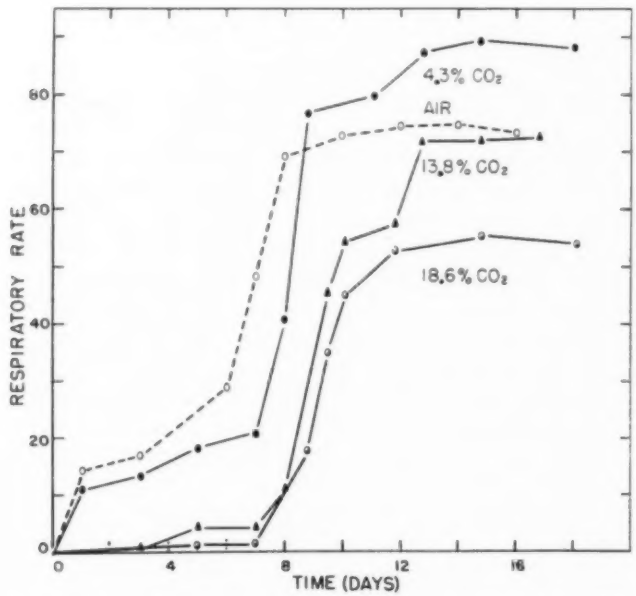

Fig. 8. Effect of carbon dioxide concentration on the respiration rate of wheat incubated at 30°C. and 18% moisture. Respiratory rate is expressed as mg. of carbon dioxide per 100 g. dry matter per 24 hours. The gas mixtures contained 21% oxygen.

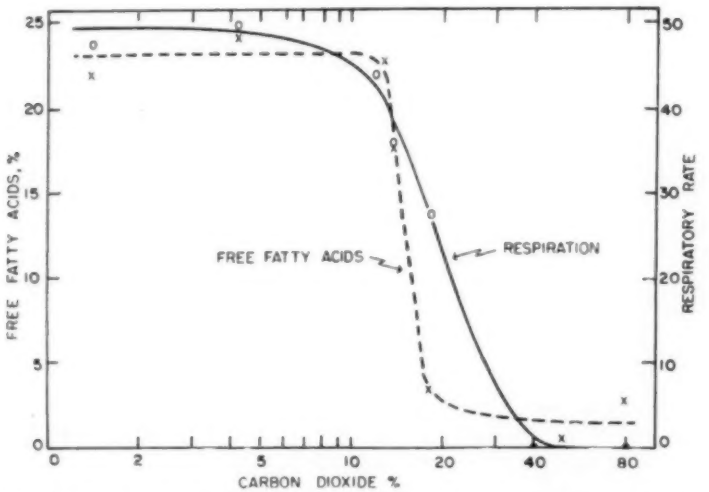

Fig. 9. Effect of carbon dioxide concentration on the mean daily respiration rate and fat acidity of wheat after incubation for 18 days at 30°C. and 18% moisture. Respiration rate is expressed as mg. of carbon dioxide per 100 g. dry matter per 24 hours. Fat acidity is expressed as mg. of potassium hydroxide per 100 g. of wheat on a 14% moisture basis. The gas mixtures contained 21% oxygen.

properties, which supports the view that the growth of various molds is a primary factor in the deterioration of stored wheat. Respiration rates and fat acidity values are quite good indexes of the extent of mold growth which has occurred. It is noteworthy that an oxygen concentration of 0.2% did not completely inhibit mold growth or respiration; at this low oxygen concentration, a high respiratory quotient indicative of anaerobic metabolism was observed. The better retention of viability and the much lower development of germ damage at this low oxygen concentration are not in accord with the view that germ-damaged or "sick" wheat results from storing wheat of high moisture content under anaerobic conditions (7, 16).

The magnitude of the effects of changes in carbon dioxide concentration on mold growth and the various properties of wheat is in striking contrast to that observed with changes in oxygen concentration. Gas mixtures containing the amount of oxygen normally present in the air had little influence on mold growth and biochemical properties until the carbon dioxide concentration was increased to the range of 13.8 to 18.6%, above which there was very marked inhibition of mold growth, of respiration, and of development of fat acidity. Higher concentrations of carbon dioxide were required to reduce the rate of loss in viability and to decrease the extent of germ damage than to retard mold growth.

Although further studies with gas mixtures containing various concentrations of oxygen and carbon dioxide are necessary, these observations appear to be of considerable practical significance in relation to grain storage. In the hermetic storage of damp grain, the decrease in oxygen concentration and the accompanying increase in carbon dioxide content of the interseed atmosphere, as a result of the respiration of the grain and associated microorganisms, will inhibit mold growth and thereby greatly retard deterioration. However, the observation that anaerobic metabolism occurs when the oxygen becomes nearly depleted leads to doubt whether damp wheat destined for human food can be stored in this manner, as the fermentation changes would produce by-products with undesirable odors and flavors. Anaerobic conditions will not prevent fermentation due to yeasts, and experiments are needed to determine the combined effect of low oxygen and high carbon dioxide concentrations on the growth of microorganisms.

Acknowledgment

This investigation was supported by a grant from the Millers' National Federation and the National Grain Trade Council.

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THE INTRINSIC VISCOSITIES OF THE WATER-SOLUBLE COMPONENTS OF WHEAT FLOUR[1]

DOYLE C. UDY[2]

ABSTRACT

Viscosity measurements on the water extracts of several wheat flours have shown that about 95% of the intrinsic viscosity of the extract is due to the polysaccharides present. The intrinsic viscosity of the soluble proteins was estimated by an indirect calculation, and they contribute the remainder of the intrinsic viscosity to the extract.

The polysaccharides and proteins are present in approximately equal amounts and constitute about 40% of the total solids. The remainder of the solids are constituents of low molecular weight which contribute very little, if at all, to the viscosity of the extract solution.

A large variation in both size and amount of soluble polysaccharides was found among many pure wheat varieties of widely differing protein content and growth environment. Single varieties were fairly consistent in these properties, and some correlation is evident between these properties and cookie diameters used in quality testing.

It has been shown (8) that the gluten proteins from different varieties have equal intrinsic viscosities[3] when dispersed in dilute acetic acid along with a very small amount of thioglycolic acid. Yet similar measurements have indicated that large viscosity differences exist in dilute acetic acid extracts of dispersible flour components from different wheat varieties at equal protein concentrations (see Fig. 1). It is reasonable to expect, therefore, that the water-soluble proteins or some other component, or both, are responsible for the viscosity differences observed in whole flour extracts. The measurements reported in this paper show the relative contribution of the soluble proteins and other water-soluble constituents to the over-all viscosity of aqueous extracts of flour.

Materials and Methods

Many pure varieties of Pacific Northwest wheats, grown in different locations and under different climatic conditions, served as an ample source for selection of samples. Straight-grade flours from these wheats, obtained with a Buhler mill, were used in all the experiments.

[1] Manuscript received June 24, 1955. Contribution from the Western Wheat Quality Laboratory, Department of Agricultural Chemistry, Washington Agricultural Experiment Stations, State College of Washington, and the Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture. Scientific Paper No. 1413, Washington Agricultural Experiment Station, Pullman.

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[3] Intrinsic viscosity is defined as $\lim_{c \to 0} (\ln \eta_r)/c$, where $\ln \eta_r$ is the natural logarithm of the relative viscosity, and c is the concentration of solute in g/100 ml of solution.

Several procedures were attempted in the preparation of suitable samples for viscosity measurements. Pence and Elder (5) prepared soluble proteins by fractional precipitation with ammonium sulfate. Although protein samples fairly free of polysaccharides were obtained by this technic, much uncertainty remained as to whether they were representative. These uncertainties arose from the fact that a considerable quantity of the originally soluble protein was lost in the fractionation procedure. Denaturation of protein during dialysis plus that remaining in solution with the polysaccharides accounted for the major protein losses.

Adsorption of the proteins on an activated clay, as used by Pence *et al.* (6) for recovery of pentosans, proved useful for estimating the intrinsic viscosity of soluble proteins by an indirect approach. Also, by this scheme the intrinsic viscosity of the polysaccharides could be measured directly. These intrinsic viscosity values provided a check for measurements made by another procedure which involved the direct precipitation of both proteins and polysaccharides. The following is a detailed description of the technics which were used.

Removal of Proteins by Adsorption. A 50-g. sample of flour in 200 ml. of distilled water was agitated in a Waring Blendor for 4 minutes, and the extracted solubles recovered by centrifuging. The extract was made 0.05*N* with respect to acetic acid prior to heating. This prevented the protein from precipitating out. Heating in a vented, stoppered test tube for 15 minutes in boiling water virtually stopped all enzyme activity. Unless deactivated, these enzymes cause moderately fast degradation of both proteins and polysaccharides. The solids concentration, along with the flow time through a Ubelohde Viscometer, was measured on an aliquot of the original solution and on subsequent dilutions of it. The original extract had approximately 1% total solids in solution. From these measurements the intrinsic viscosity was determined as described in an earlier report (8).

Special Filtrol[4] was added to the remaining solution in the amount of 6 g/100 ml. Shaking the solution with added Filtrol for approximately 30 minutes before centrifuging effectively removed about 85% of the total nitrogen. The intrinsic viscosity of the constituents left in this centrifuged solution was then determined. This value was used in conjunction with the intrinsic viscosity, determined before removal of any protein, to calculate the intrinsic viscosity of the protein by the following equation:

$$[\eta] = \sum_{i}^{n} [\eta]_i w_i$$

[4] Special Filtrol is one of several activated clays marketed by the Filtrol Corporation, Los Angeles, California.

where $[\eta]_i$ is the intrinsic viscosity of fraction i and w_i is its weight fraction. The summation is made over all n fractions. A fraction may include any amount or number of individual constituents. In the computation of the intrinsic viscosity of protein, all constituents exclusive of the removed protein constitute one fraction; the removed protein constitutes the other fraction. This equation is used for fractionated high polymers (4), and its validity has been demonstrated here with independent and combined samples of gluten protein and polysaccharides. Values measured directly on the combined fractions are in good agreement with the values calculated from the foregoing equation.

Protein (*Wt. Fraction*)	*Polysaccharides* (*Wt. Fraction*)	$[\eta]$	
		(*Measured*)	(*Calculated*)
0.0	1.0	4.30	...
1.0	0.0	0.19	...
0.362	0.638	2.68	2.81
0.604	0.396	1.78	1.81
0.909	0.091	0.55	0.56

The next step was to dialyze the Filtrol-treated solution for 4 days, which removed essentially all of the components of low molecular weight. The intrinsic viscosity of the soluble polysaccharides was determined from this dialyzed solution after correction for the small amount of protein ($N \times 5.7$) remaining.

An alternative to the dialysis step, for eliminating the components of low molecular weight, was to precipitate the polysaccharides and remaining protein with acetone. This was accomplished by adding two volumes of acetone to one volume of Filtrol-treated extract. The resulting precipitate, now free from low-molecular-weight components, was redissolved in distilled water and the intrinsic viscosity of the polysaccharides in the solution determined. A small correction is again required for the remaining protein. Agreement with the dialysis technic was satisfactory (Table I).

Direct Precipitation of Proteins and Polysaccharides. The procedure used for determining the intrinsic viscosity of the polysaccharides, as recorded in Table II, is more indirect than the method described above. After the acidified extract was heated and its intrinsic viscosity and nitrogen content were determined, the polysaccharides and protein were precipitated directly by adding two volumes of acetone to one volume of extract. The precipitate was dried and weighed, and the amount of protein ($N \times 5.7$) left in the supernatant determined. The nonprotein nitrogen in the original aqueous extracts of flour varied from 2 to 5% of the total nitrogen. Nearly half of the nitrogen remained in the supernatant liquid, indicating that these proteins have

a rather low molecular weight. From these data the percent of polysaccharides, proteins and low-molecular-weight constituents in the original extract were calculated. A small correction for the contribution of protein to the over-all intrinsic viscosity permitted calculation of the intrinsic viscosity of the polysaccharides by use of the equation cited previously. A value of 0.20 for the intrinsic viscosity of soluble protein was used in calculating the intrinsic viscosity of the polysaccharide fraction by the direct precipitation method.

Results and Discussion

A comparison of the values obtained by the protein adsorption and direct precipitation technics is given in Table I.

TABLE I

INTRINSIC VISCOSITIES OF POLYSACCHARIDES AS DETERMINED BY THREE DIFFERENT PROCEDURES AND OF THE SOLUBLE PROTEINS BY A SINGLE INDIRECT CALCULATION

WHEAT VARIETY	WHEAT TYPE	POLYSACCHARIDE FRACTION			SOLUBLE PROTEIN FRACTION (CALCULATED)
		Filtrol-Treated		Direct Acetone Precipitation	
		Dialysis	Acetone Precipitation		
Idaed	SW	2.8	...	2.8	0.26
		2.5	...	...	0.15
Rio	HRW	4.1	...	4.3	0.22
		4.1	...	...	...
Golden	SW	2.4	...	2.8	0.20
Kharkof	HRW	3.8	3.7	4.1	0.15
		...	4.0	...	...
Elmar	Club	2.7	3.1	3.3	0.24

The agreement in intrinsic viscosity obtained by direct precipitation, as compared with the other procedures, is sufficiently satisfactory to warrant its use for comparing soluble polysaccharide fractions obtained from several different varieties of wheat. Results of measurements made by this procedure are recorded in Table II.

Although the intrinsic viscosity of the soluble proteins, as calculated by the foregoing indirect procedure, is not accurate enough to make comparisons among varieties, it does suffice to give an approximate value. When gluten is dispersed in dilute acetic acid in the presence of a small amount of reducing agent, it has an intrinsic viscosity of 0.19 (8). As shown in Table I, the intrinsic viscosity of the soluble proteins, calculated by the indirect method outlined above, varied from 0.15 to 0.26. Since these soluble proteins are considered to be lower in molecular weight than gluten, these values would appear reasonable. Accuracy is limited by the fact that the soluble pro-

teins contribute only about 6% of the over-all intrinsic viscosity of the water-soluble extract of flour.

The most interesting results were obtained in the determination of the intrinsic viscosity of the soluble polysaccharide fraction of several flours, milled from varieties containing various flour protein levels. The data are in Table II.

TABLE II

DISTRIBUTION OF COMPONENTS IN WATER-SOLUBLE EXTRACTS OF FLOUR AND THE INTRINSIC VISCOSITY OF THE POLYSACCHARIDE FRACTION FOR SEVERAL VARIETIES

Wheat Variety	Wheat Type	Protein in Flour	Polysaccharides in Extract	Proteins in Extract	Dialyzables in Extract	$[\eta]_{ps}$ [a]	$[\eta]_{ps} \times w_{ps}$ [b]
		%	%	%	%		
Rio	HRW	6.5	23.1	18.4	58.5	4.4	1.02
		8.1	23.6	17.6	58.8	4.4	1.04
		9.5	28.3	16.6	55.1	4.3	1.22
		13.2	26.8	22.9	50.3	4.4	1.18
Kharkof	HRW	6.6	24.6	20.0	55.4	4.1	1.01
		7.8	22.5	19.6	57.9	3.9	0.87
		12.9	23.7	24.7	51.6	4.3	1.02
Wasatch	HRW	9.4	22.0	17.5	60.5	2.9	0.64
		13.1	23.4	26.8	49.8	3.8	0.87
		12.0	21.5	23.9	54.6	3.0	0.65
Brevor	SW	5.7	18.0	24.5	57.5	2.8	0.50
		5.9	16.2	17.0	66.8	2.9	0.47
		10.9	16.7	25.2	58.1	2.9	0.49
Golden	SW	6.1	17.3	22.4	60.3	2.8	0.48
		6.5	17.1	19.5	63.4	3.1	0.53
		11.6	17.7	26.8	55.5	3.3	0.59
Rex	SW	7.4	18.7	21.6	59.7	2.9	0.54
		12.0	14.3	17.5	68.2	2.7	0.39
		12.9	16.4	27.1	56.5	3.2	0.53
Elmar	Club	5.9	20.5	23.7	55.8	3.3	0.68
		6.6	19.4	20.9	59.7	2.8	0.55
		12.5	20.5	30.1	49.4	3.7	0.77
Elgin	Club	8.2	18.5	21.0	60.5	2.8	0.52
Marfed	SW	7.3	21.1	21.1	57.8	3.5	0.74
Awned							
Onas	SW	9.3	19.5	23.4	57.1	3.2	0.62
Baart	SW	8.1	19.1	22.6	58.3	3.3	0.63
Idaed	SW	8.1	18.7	26.8	54.5	2.8	0.52

[a] Intrinsic viscosity of the polysaccharide fraction.
[b] Product of the weight fraction and intrinsic viscosity of polysaccharides in the original extract. Referred to as viscosity index in the text.

The intrinsic viscosity value for the polysaccharides is 15- to 20-fold larger than for the soluble proteins. It is readily apparent that the polysaccharides dominate the viscosity differences referred to in the introduction and portrayed in Fig. 1. The greater relative amount and size of soluble polysaccharides in Rio flour account for the in-

creased viscosity over that of Elgin flour. On the other hand, the proteins of each variety contribute equally at a given protein concentration. The total proteins contribute about one-third of the total intrinsic viscosity.

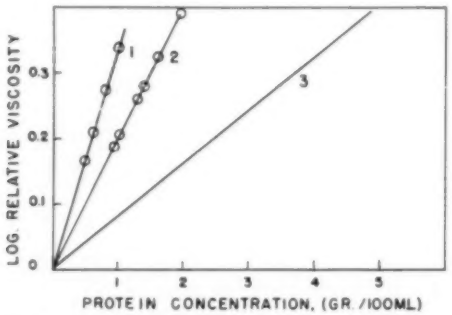

Fig. 1. Dependence of relative viscosity on protein concentration. Curves 1 and 2 are for dispersed proteins and other soluble components of flour from Rio and Elgin varieties, respectively, in a 0.05*M* acetic acid — 0.005*M* thioglycolic acid aqueous solvent. Curve 3 is for dispersed gluten from any variety in the same solvent system (taken from reference 8).

Salts and other components of low molecular weight comprise about 60% of the total soluble solids. They do not, however, contribute to the intrinsic viscosity. This is somewhat surprising in view of experimental results associating flour paste viscosity with ash content (2, 3). A possible explanation for these results may be primarily in the method of viscosity measurement itself. Viscosities of acidulated flour suspensions are unstable and exhibit thixotropic properties. It is reasonable to expect, and Bresson and Barmore have shown (2), that ash and protein exert considerable effect on viscosity and are undoubtedly responsible for part of this unstable structural viscosity. On the other hand, the intrinsic viscosities, as measured here, are determined from dispersions or solutions having stable viscosities, and the over-all intrinsic viscosity of a flour extract is the sum of the products of intrinsic viscosity and weight fraction for each individual component. The presence of any one component or a combination of components does not influence the contribution of a given component to the over-all solution viscosity.

Dispersions of flour in water and lactic acid exhibit unstable structural viscosities (as measured in MacMichael units) as much as tenfold greater than can be accounted for by the dispersed proteins and polysaccharides. These facts must be explained by other phenomena. The soluble polysaccharides account for approximately two-thirds of the normal stable intrinsic viscosity of flour extracts, and the dispersed

soluble and gluten proteins supply the remaining one-third. This is even more impressive when it is recalled that an average flour of 10% protein content has only about 1% soluble polysaccharides.

Another common observation is the positive relation between ash content and the viscosity of acidified dispersions of different mill streams. This may possibly be explained by the physical association of inorganic substances with soluble polysaccharides within the varied layers of the wheat kernel. For example, a specific area of the kernel which accumulates in a single mill stream may be high in both ash and soluble polysaccharides. Also, the amount of polysaccharides that will be soluble is related to the extent and degree of grinding.

It is significant to note that the intrinsic viscosity of a polysaccharide extract of wheat flour prepared and measured by Perlin (7) was 3.4. This extract was made up of both hexosans and pentosans and should be comparable to the soluble polysaccharide extracts described here.

Some significance should probably be attached to the fact that the bread type varieties Rio and Kharkof have a high viscosity index, as shown in the last column of Table II. These varieties produce cookies of very small diameter. The cookie or soft types, on the other hand, have a low viscosity index. Cookie diameters for several of the varieties used in this study have been repeatedly measured and show a fairly close relation with the viscosity index. Yamazaki (9) recently has shown a high correlation between cookie diameters and alkaline water retention capacity. Although proteins imbibe considerable water, differences in the water uptake of a good and a poor cookie flour at the same protein level are presumably the result of differences in amount and size of the polysaccharides which imbibe water. The degree of swelling or solvent uptake in high polymers is related to their size and structure.

The protein content of the flour does not appear to affect either the amount of soluble polysaccharides present or their size. This is shown by the constancy of the intrinsic viscosity value within a given variety. Consequently, the average size of the soluble polysaccharide molecules is a specific varietal characteristic. The tendency for greater or lesser amounts of soluble polysaccharides to be extracted from wheat flours also follows varietal types rather closely.

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RELATIONS BETWEEN WHEAT PROTEIN CONTENT, LOAF VOLUME, EXPANSION VOLUME, AND SEDIMENTATION VALUE. I.
Plant Breeders' Samples[1]

R. H. HARRIS AND L. D. SIBBITT[2]

ABSTRACT

Protein content, expansion volume, sedimentation value, and loaf volume were determined for 50 wheat hybrids grown in Mexico and 55 grown in North Dakota. In the Mexican series, the closest correlation with loaf volume was obtained with sedimentation value (0.78**), followed by expansion volume (0.60**) and protein content (0.54**). Partial correlation coefficients with loaf volume, independent of protein content, were 0.70** for sedimentation value and 0.66** for expansion volume. For the North Dakota series, which were of much higher baking strength, the only significant correlation was a negative one between loaf volume and protein content. When flours from the North Dakota series were baked with an equal part of soft flour, a significant correlation was obtained only for loaf volume and sedimentation value (0.60**). Expansion volumes, and especially sedimentation values, may have some use for screening promising materials from collections of hybrid lines of widely varying genetic origin.

This study was initiated to discover whether a series of wheat samples differing widely in protein content and baking strength could be at least partially evaluated in respect to loaf volume by their expansion volume (3, 4) or their sedimentation value (6). If so, the number of samples requiring baking might be reduced by discarding those

[1] Manuscript received May 26, 1955. Published with the approval of the Director, North Dakota Agricultural Experiment Station, Fargo, North Dakota.
[2] Department of Cereal Technology, North Dakota Agricultural Experiment Station.

samples with low expansion or sedimentation values and baking only the remainder with resulting economy in time and material.

Materials and Methods

The wheats used were two series of plant breeders' samples which were submitted for milling and baking quality tests by micro methods (1, 5); one consisted of 50 samples grown in Mexico and the other of 55 samples of hard red spring wheat from a North Dakota nursery. Both series contained hybrids of markedly diverse parentage and the Mexican series, as expected, proved to be more variable in quality than nursery or plot samples which have passed preliminary quality tests. The North Dakota series was generally much higher and less variable in baking strength than the Mexican, although they were still in an early stage of selection.

The flours were baked by the malt-phosphate-bromate formula with 3 hours' fermentation and 25 g. of flour (2). A National micro mixer was used for mixing. For the hard red spring series a blend of 50% by weight of soft wheat flour of 8.6% protein content was also baked in an attempt to bring out the potential strength of these high-protein samples. Only 45 residues from the hard spring samples were available for these blend bakings.

The procedure for the expansion test was that described by Miller, Edgar, and Whiteside (4), except that 10 g. instead of 5 g. of flour were mixed with the micro mixer, and the resultant dough divided into equal halves by weight. Absorptions were as used for baking. Sedimentation values were obtained as described by Zeleny (6) with special attention to shaking technic.

Results

Table I shows the means, the standard deviations of the population, and the coefficients of variation for the different analytical data obtained on the two series of hybrid wheats. All the values were markedly lower for the Mexican wheats, particularly the sedimentation results, than for the hard spring wheats. However, the variability was much higher for the Mexican series. Blending the hard red spring flours with 50% soft wheat reduced the mean loaf volume 45 cc., and the coefficient of variation from 9.6 to 7.0%.

Table II provides information on the relationships between the quality determinations for both series. The correlation coefficient between wheat protein content and expansion volume, and that between wheat protein content and specific sedimentation value, were not significant. For the Mexican wheats, expansion volume appeared to be measuring the quality rather than the quantity of protein. While the

TABLE I

STATISTICAL DATA CALCULATED FROM THE HYBRID WHEATS

STATISTIC	WHEAT PROTEIN	LOAF VOLUME	EXPANSION VOLUME	SEDIMENTATION VALUE	SPECIFIC SEDIMENTATION VALUE
Mexican wheats					
Mean	12.8%	161 cc.	12.4 ml.	30.8	2.4
Standard deviation	1.71	21.51	3.22	9.60	0.64
Coefficient of variation, %	13.4	13.4	30.0	31.2	26.7
Hard red spring wheats, malt-phosphate-bromate method					
Mean	15.8%	225 cc.	18.0 ml.	60.8	
Standard deviation	0.77	21.66	1.86	7.50	
Coefficient of variation, %	4.9	9.6	10.3	12.3	
Micro-blend method					
Mean	16.0%	180 cc.	18.0 ml.	61.0 ml.	
Standard deviation	0.48	12.65	1.92	7.26	
Coefficient of variation, %	3.0	7.0	10.7	11.9	

remaining correlations were highly significant, none was sufficiently large to allow very accurate prediction of loaf volume. However, any method that will assist in removing from a large number of hybrid lines a proportion of lines having lower average quality than the remainder will enable the breeder to concentrate his efforts on the more promising material.

Sedimentation value yielded the largest correlation coefficient with loaf volume, although not significantly higher than for expansion volume and loaf volume. It was significantly higher than for wheat protein and loaf volume. Generally, the relations between expansion volume

TABLE II

CORRELATION COEFFICIENTS FOR THE HYBRID WHEATS

VARIABLES CORRELATED	MEXICAN WHEATS (N = 50)	HARD RED SPRING WHEATS	
		Malt-Phosphate-Bromate (N = 55)	Blend (N = 45)
Wheat protein and loaf volume	+0.537**	−0.402**	−0.131
Wheat protein and expansion volume	+0.078	−0.187	+0.108
Wheat protein and sedimentation value	+0.482**	+0.228	−0.233
Expansion volume and loaf volume	+0.601**	+0.101	−0.365*
Sedimentation value and loaf volume	+0.780**	+0.226	+0.599**
Expansion volume and sedimentation value	+0.675**	+0.048	−0.068
Partial Correlation:			
Expansion volume with loaf volume, wheat protein constant	+0.665**		
Sedimentation value and loaf volume, wheat protein constant	+0.705**		

and loaf volume are in good agreement with those of Miller, Edgar, and Whiteside (3), falling approximately midway in magnitude between their Lethbridge and Winnipeg series. For the Mexican series a very significant gain of information on loaf volume was obtained from expansion volume or sedimentation value data in addition to that obtained from a knowledge of wheat protein content. The multiple correlation coefficient for the relationship of loaf volume to the combined effect of expansion volume and protein content was +0.792. The corresponding value for the relationship of loaf volume to the combined effect of sedimentation value and protein content was +0.801. The removal of the influence of wheat protein from the correlations of expansion volume and sedimentation value with loaf volume by partial correlation technic had little effect; the greatest change was with sedimentation value and loaf volume, where the correlation became 0.705.

None of the three methods — wheat protein content, expansion volume, or sedimentation value — was by itself of marked value for preliminary sorting out or screening the Mexican hybrids for baking quality. In glancing over the data it was possible to pick out a few samples with markedly low expansion and sedimentation values which had unsatisfactory loaf volume, but in the majority of these instances the protein content was also below a level at which acceptable loaves would be expected, with comparatively few samples eliminated by these two tests.

The lack of significant positive correlation between loaf volume and other quality properties for the malt-phosphate-bromate method in the North Dakota wheat series was probably caused by the high level of strength in these hybrid wheats, and determinations of wheat protein content, flour expansion volume, or sedimentation value would not assist in differentiating them without using the baking test. For the blend bakings there was a highly significant positive relation between sedimentation value and loaf volume. A baking test on each sample would apparently be required to evaluate this series of hybrid wheats.

From the expansion volume test, as pointed out by Miller *et al.* (4), some concept may be obtained of the flour absorption and mixing requirements and the handling properties of the dough. Also, the appearance of the dough ball after the 45-minute preliminary fermentation just before it is placed in the expansion apparatus yields some indication of the interior loaf characteristics which may be expected. This is particularly true for samples of doubtful baking quality. A coarse appearance of the surface tends to indicate poor grain and tex-

ture in the resultant loaf. The protein and sedimentation tests obviously cannot furnish this information. However, the sedimentation test requires even less flour than the expansion procedure, is much simpler, and does not require the equipment needed for the expansion test. Sedimentation values appeared to be slightly more useful for identifying samples of poor potential loaf volume in the Mexican, than in the North Dakota series, probably because of their higher correlation with loaf volume than that between wheat protein content and loaf volume. The use of specific sedimentation value did not offer any advantage in this series since its relation to loaf volume was not greater than that for sedimentation value uncorrected for protein content.

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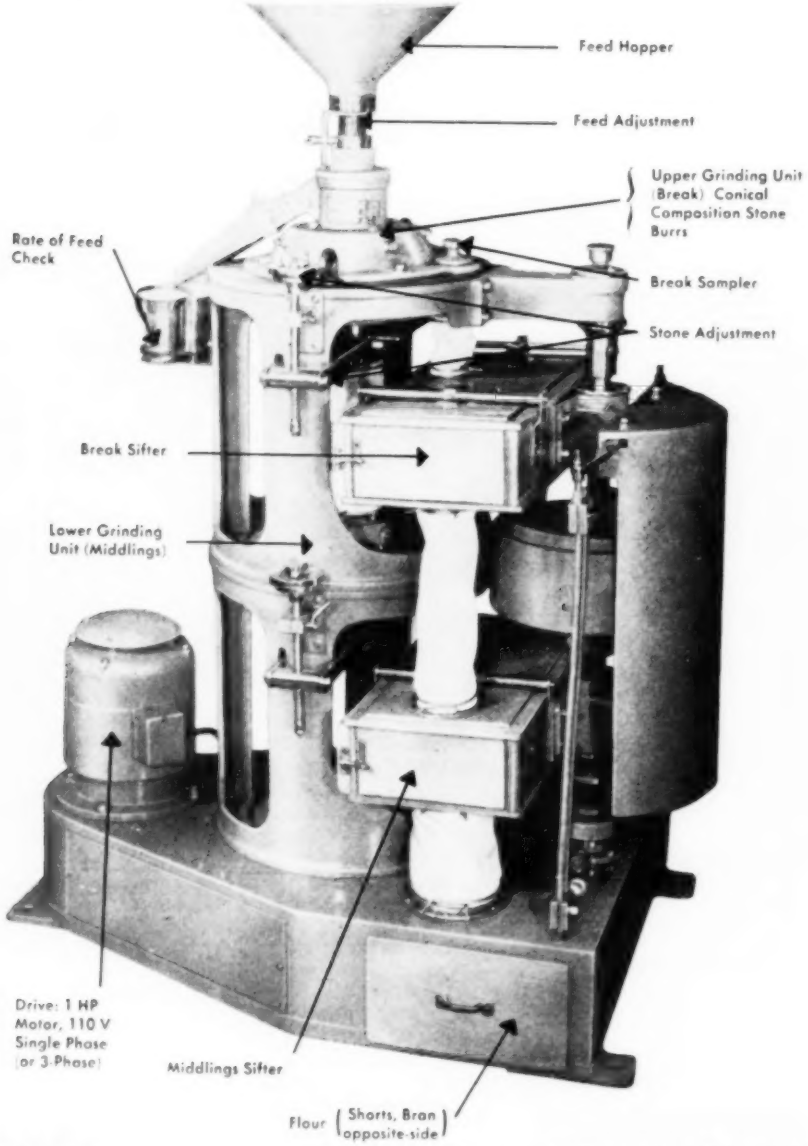

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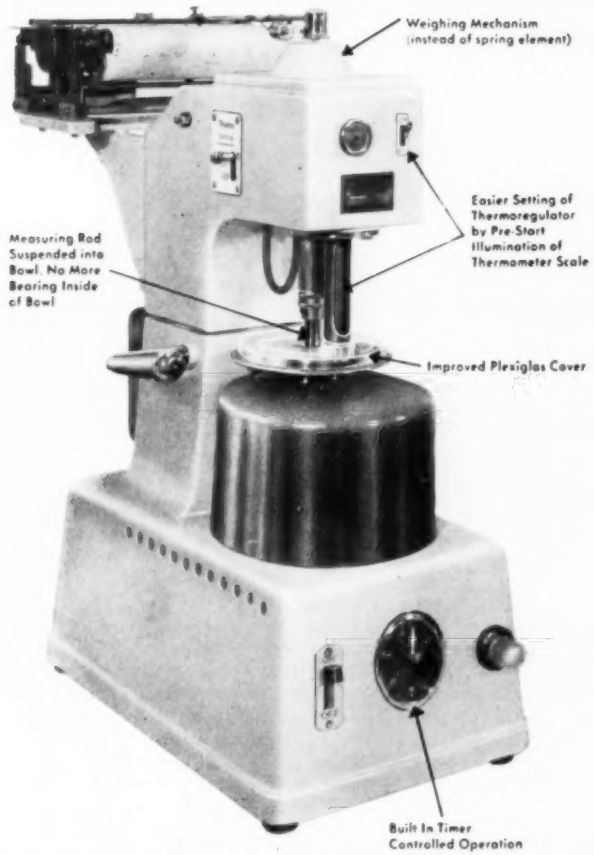
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The American Association of Cereal Chemists is a professional organization of people engaged in these areas in the United States, Canada, and elsewhere, and as such does not have funds to finance such a program. It is hoped that the institutions, the governments, and organizations sponsoring such research might find it possible to send people to this international symposium.

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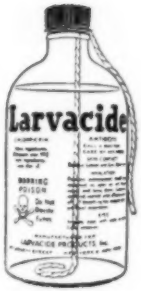

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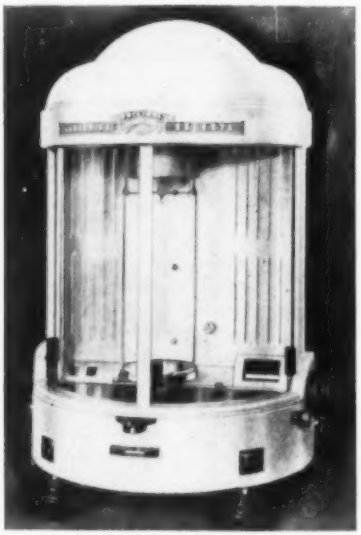

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when you use . . .

THE DYOX PROCESS® for flour maturing . . .

The fixed uniformity of DYOX treated flour is a definite advantage to the miller who strives to meet the baker's demand for a starting product of constant value—which works in the bake shops with the least trouble and manipulation by the operator. DYOX treated flour assures overall dependability, uniformity of product, and ease of operation.

"NOVADELOX"® for whiter, brighter flour . . .

"Novadelox" is designed to meet the desire of the consuming public for a bread that is truly white—and to enable millers to produce a uniform product of standard color.

"N-RICHMENT-A"® for uniform enriching . . .

"N-Richment-A" provides a readily available, simple and troublefree product for enriching flour—with the assurance that the standard procedure of adding "N-RICHMENT-A" will produce a dependable standardized flour.

THE N-A FLOUR SERVICE DIVISION with more than a quarter-century of experience . . .

The N-A Flour Service Division with its skilled laboratories and staff is always available to work with you or your consultants on all phases of maturing, bleaching and enriching. Why not phone your nearest N-A Representative today!

DYOX
for flour maturing
NOVADELOX
for a whiter, brighter flour
N-Richment-A
for uniform enrichment

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NOVADEL FLOUR SERVICE DIVISION

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